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Studies on yeast invertase

II. Inactivation of invertase by iodine and by heavy metal ions[1]

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With 9 figures in the text

The peculiar action of free iodine on invertase was discovered by v. Euler and Landergren (1). In contrast to chlorine and bromine which, in very low concentrations, cause a rapid and complete loss of the enzymatic activity of invertase (2, 3, 4), iodine inactivates invertase in a two-step reaction: On the addition of small amounts of iodine to a yeast invertase solution the activity decreases very rapidly to about 55 % of the original value, irrespective, in rather wide limits, of the amount of iodine added. However, if iodine is added in large amounts and is allowed to act on the enzyme for longer periods of time before the activity determination, a second slow reaction phase is observed. Complete inactivation may result after long incubation times if enough iodine has been added. The velocity of this second phase is dependent on the pH of the solution; a minimum is found in the range pH = 4–5, i.e. in the region of optimal activity of the enzyme (4). The velocity of the first rapid phase is so high that no accurate values can be determined; this phase of the reaction seems to be fairly independent of the pH, no marked differences being found in the range pH = 2.6 –6.5. The difference in the velocities of the two phases is so great that in experiments with short reaction time the same relative activity, (RA) = 0.55, is found even if iodine is added in great excess (cf. fig. 1). If necessary, the reaction may be stopped after the first phase by the addition of some suitable agent, reacting rapidly with iodine and having no action on the enzyme itself. Cysteine, for example, may be used but not thiosulphate (4).

The results obtained (1–4, 5) concerning the action of iodine on yeast invertase are explained in the following way: In the first rapid phase an invertase derivative is produced, the specific activity of which is about 55 % of that of the native enzyme; in the second, slow phase this derivative reacts with more iodine, eventually yielding a quite inactive secondary derivative. In the following the product with RA = 0.55 will be indicated as "I-invertase" (5). This designation should not necessarily intimate that I-invertase contains iodine; the nature of the interaction between enzyme and iodine is a matter of dispute. Experiments have been carried out (6) using radioactive iodine in order to find out if organically bound iodine is present in I-invertase. However, since yeast invertase is not known in pure form and the amount and nature of impurities in the invertase preparations cannot be stated, the results were ambiguous.

As a tentative explanation of the partial inactivation of yeast invertase by iodine

[1] Part I, cf. reference (5).

v. Euler and Josephson (3) proposed the wellknown oxidation of HS- to –S–S-groups. (The authors appear to regard the fact that the specific activity of I-invertase is approximately half that of the native enzyme, as a support of this theory.) Although this explanation may appear the most probable, substitution of aromatic or heterocyclic nuclei must also be taken into consideration. The following experiments were undertaken in the hope of throwing some light on the problem.

Experimental

Enzyme.—Partially purified preparations from baker's yeast.

Activity determination.—Polarimetric determination as described earlier (7). The RA values are means of 4–6 determinations of the degree of hydrolysis at different reaction times. Temperature 30°C.

Reagents.—All reagents were p.a. grade; redistilled water was used throughout. The iodine solutions used were diluted from a 0.1 N solution of iodine in 0.1 *M* KJ solution.

Buffer.—1 M sodium acetate containing suitable amounts of acetic acid. Concentration of sodium acetate in the assay mixtures usually 0.01 *M*. The pH was determined by means of the glass electrode.

Formation of I-invertase

The activity of the invertase solutions used in the following experiments was such that 1 ml hydrolyzed 100 ml 4.75 % saccharose solution to 50 % in about 1 h (pH 4.5, 30°).

To 1 ml invertase solution +10 ml buffer, pH 4.5 + 68 ml water was added 1 ml iodine solution, containing the amounts of iodine recorded in Table 1. After incubation at 30° a solution of 4.75 g saccharose in 20 ml was added and the RA was determined. The activity values of Table 1 refer to the original activity, put = 1.

The experiments are in complete agreement with previous results. The two sharply separated phases of the reaction of invertase with iodine are clearly seen in Fig. 1.

To produce I-invertase for the experiments described in the following 0.06 mg iodine was used per ml of invertase solution. This amount of iodine was consumed in less than 1 min by the enzyme and, presumably, partly by impurities in the enzyme preparation. No secondary inactivation, i.e. no decrease of RA to values <0.55 was

Table 1. Relative activity (RA) of invertase treated with iodine.

mg Iodine added	Incubation time min.	RA
0.0195	1	0.82
0.0195	10	0.82
0.039	1	0.63
0.058	1	0.54
0.058	60	0.50
0.39	1	0.54
0.39	10	0.52
0.39	60	0.40
1.95	1	0.50
4.9	10	0.46

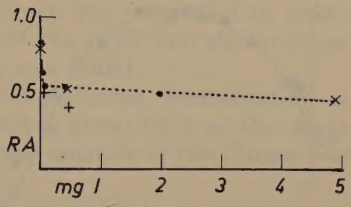

Fig. 1. Relative activity of invertase treated with iodine: ●, Incubation time 1 min.; ×, Incubation time 10 min.; +, Incubation time 60 min.

observed in these experiments. The solutions of I-invertase were dialysed to remove KJ etc. No activity losses were caused by the dialysis. The solutions of I-invertase were stable for weeks at 0–3°C. However, the stability compared to that of the parent invertase solutions appeared to be somewhat diminished.

Substrate affinity and activity-pH-curve of I-invertase

I-Invertase appears to have practically the same affinity for saccharose (i.e. the same "Michaelis' constant") as the native enzyme (4). Recent experiments support this conclusion. This would mean that the substrate-binding groups of the enzyme protein have not been affected by the treatment with iodine.

v. Euler and Josephson (3) reported that the activity-pH-curve of I-invertase seems to agree rather well with that of the native invertase. This result has been verified by new experiments (Table 2). The values have been plotted versus pH in Fig. 2. The activity at pH 4.5 has been put = 1 for both enzymes. The figure shows that the pH dependence of I-invertase is the same as that of the native enzyme; the small differences found (in the alkaline branch of the curve) are probably within the limits of experimental error. This would mean that the groups determining the activity-pH-curve of invertase are not involved in the reaction of the enzyme with iodine. It should be remembered in this connection that the acid branch of the activity-pH-curve is probably determined by a substrate-binding group (4) with $pK \sim 3$.

Attempts at reactivation of I-invertase

v. Euler and Landergren (1) found no reactivation of I-invertase by sodium thiosulphate. Further experiments (4) showed that not only is no reactivation of I-invertase achieved by thiosulphate, but the addition of thiosulphate to a mixture of I-invertase and iodine causes an extremely rapid inactivation of I-invertase. Afterwards a certain auto-reactivation can sometimes be observed. The nature of these reactions is not known. Anyway thiosulphate cannot be used to remove excess iodine from I-invertase.

If we assume that the action of iodine upon invertase, i.e. the formation of I-invertase, is an oxidation of HS-groups, it must be remembered that an oxidation of HS-groups by iodine must not necessarily yield the corresponding disulphide; an "overoxidation" may very well occur. It may be mentioned for instance that reduced glutathione (GSH) when titrated with iodine in diluted solution consumes the amount of iodine calculated for the conversion of GSH to GSSG whereas cysteine under the same conditions is very strongly overoxidized. However, since the simplest explanation of the action of iodine on invertase is an oxidation of HS-groups to the

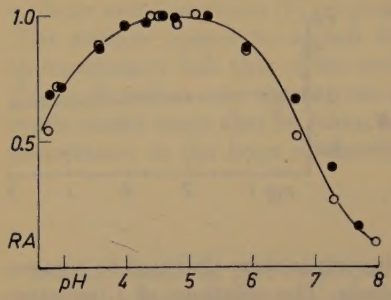

Fig. 2. pH-Dependence of invertase (O) and I-invertese (•).

disulphide level, it seemed worth while to try reactivation of I-invertase by reducing agents:

Cysteine. 1 ml I-invertase solution was incubated with 25 mg cysteine hydrochloride + 0.1 g sodium acetate for various lengths of time at 30°. No change in the enzymatic activity could be observed.

Glutahione (GSH) and *thioglycolic acid* were tried in corresponding concentrations in similar experiments; no action was found.

Ascorbic acid did not have any perceptible action.

Sodium dithionite. 1 ml I-invertase solution was treated with 20 mg dithionite in acetate buffer at pH 5 and 30°. No reactivation of the enzyme was observed. *Sulphites* likewise had no influence on the enzyme activity.

Sodium amalgam. 5 ml I-invertase solution +0.35 g $NaHCO_3$; a slow stream of CO_2 was led through the solution and ca 10 g amalgam (0.2 g Na) was added in portions during 20 min. No increase of the activity could be noticed. Control experiments with native invertase showed that the enzyme was not damaged by the same treatment.

Sodium borohydride. 2 ml I-invertase solution, 0.1 g $NaHCO_3$, 25 mg $NaBH_4$. Experiments at 0° and 20°; time 20 or 30 min. No reactivation was observed and controls showed that the native enzyme was not affected by the same treatment.

Table 2. RA of invertase and I-invertase at various pH values.

Invertase		I-invertase	
pH	RA	pH	RA
2.78	0.54	2.75	0.69
2.90	0.72	2.89	0.72
3.48	0.88	3.48	0.87
4.40	1.00	3.97	0.96
4.51	1.00	4.27	0.97
4.79	0.96	4.51	1.00
5.10	1.00	4.78	0.99
5.92	0.85	5.32	1.00
6.67	0.54	5.94	0.87
7.26	0.26	6.66	0.67
7.98	0.10	7.25	0.40
		7.68	0.16

"*Phytochemical reduction.*"—(*a*) 2 g fresh baker's yeast was suspended in 10 ml I-invertase solution. The suspension was filtered after 20 min at 30° and the invertase activity of the filtrate was determined. No increase was found.

(*b*) 2 g baker's yeast, 10 ml I-invertase solution, 0.2 g glucose. Fermentation at 30°; evolution of CO_2 was measured. After 20 min. when about 90 % of the sugar was fermented the solution was filtered and the invertase activity of the filtrate was determined; no change was observed.

Assuming that the partial inactivation of invertase by iodine is an oxidation of HS-groups of the enzyme molecule, the above negative experiments may be explained in at least two different ways:

(*a*) The treatment of invertase by iodine may cause an overoxidation, i.e. may yield products with a higher oxidation level of the sulphur than the disulphide. Such products may not be reducible by the agents used in the experiments.

(*b*) If the oxidation of the enzyme by iodine results in the formation of disulphidic bonds we have to assume, either that these bonds are not reduced under the experimental conditions applied or, perhaps more probable, that reduction of the disulphidic bonds does not cause a complete reconstitution of the structure of the native enzyme. It is of course quite possible that oxidation of HS– to S–S-groups causes appreciable changes in the "tertiary" structure of the enzyme protein and that mere reduction of the disulphidic bonds is not accompanied by a complete rearrangement to the original structure of the native protein.

As pointed out before, oxidation of HS-groups is not the only possible explanation of the action of iodine on invertase.

It may be mentioned that I-invertase, like the native invertase, it not precipitated by saturation of the solution with ammonium sulphate. Neither I-invertase nor the native enzyme are attacked by papain.

Inhibition of I-invertase by heavy metal ions

Since mercaptide formation with essential HS-groups of the enzyme protein may be an acceptable explanation of the inhibition of invertase by mercury compounds and possibly by Ag- and certain other heavy metal ions, it was of interest to investigate the behaviour of I-invertase towards inhibitors of this kind. As pointed out earlier (4, 8–10), the inhibition of invertase by Ag^+, Cu^{++}, Pb^{++}, Zn^{++}, Cd^{++}, and UO_2^{++} shows a very characteristic dependence upon the pH of the medium, whereas the inhibition by $HgCl_2$ and organic mercury compounds (11) is rather independent of pH. If the mercury compounds are supposed to react with HS-groups of the enzyme protein—and nothing known at present appears to contradict this assumption—then we have to assume that Ag^+, Cu^{++} etc. are bound, either by non-HS-groups or at the same time by HS- and non-HS-groups, or to HS-groups remarkably different in their chemical behaviour from the ordinary HS-groups. In view of the possibility that the action of iodine on invertase is an oxidation of HS-groups experiments with I-invertase and heavy metals were carried out.

The preparation of I-invertase involves introduction of iodide ions into the solutions. Obviously these have to be removed before the experiments with metal ions, especially those with Ag^+. The iodide ions were removed by exhaustive dialysis of the I-invertase solutions against redistilled water that was changed several times. Control experiments in which fairly large amounts of potassium iodide were added to native invertase before dialysis showed that the iodide ions could be effectively removed in this way; the dialyzed, native enzyme was inhibited by Ag^+ in exactly the same way as the enzyme solution before addition of KI.

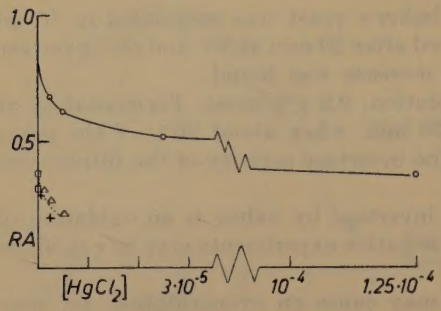

Fig. 3. Inhibition by $HgCl_2$: O, I-invertase; +, native enzyme; △, native enzyme, earlier experiments; □, earlier experiments with highly purified native enzyme.

Action of $HgCl_2$ on native invertase and I-invertase.—The experiments were carried out as described earlier (11). The solutions of native enzyme used in the controls was diluted to the same activity as that of the I-invertase solution. Experiments with various concentrations of $HgCl_2$ (Table 3) and two different preparations of I-invertase are illustrated by Fig. 3, in which also some earlier experiments with native invertase and $HgCl_2$ are included. The figure shows clearly that I-invertase is considerably less affected by the inhibitor than is the native enzyme. 50 % inhibition of the native enzyme is found at a concentration of $HgCl_2 \sim 5 \times 10^{-7}$ *M* (without previous incubation of enzyme and inhibitor, and at still lower concentrations with incubation (11)); 50 % inhibition of I-invertase requires a $HgCl_2$ concentration of at least 3×10^{-5} *M*, i.e. about the 100-fold concentration. Obviously this can be explained by the assumption that in the native enzyme HS-groups are responsible for the binding of Hg and that in the I-invertase these groups are in an oxidized and non-reactive form. The comparatively very low affinity of I-invertase for Hg would intimate that in the native enzyme the HS-groups are essentially the only groups that react with low concentrations of Hg salts.

Table 3. Action of $HgCl_2$ on I-invertase and native invertase at pH 5.3.

I-invertase No. 1			I-invertase No. 2		
$[HgCl_2]$ mole/l	Incubation, min.	RA	$[HgCl_2]$ mole/l	Incubation, min.	RA
2.5×10^{-6}	0	0.68	2.5×10^{-5}	0	0.65
5×10^{-6}	0	0.63	2.5×10^{-5}	30	0.52
2.5×10^{-5}	0	0.55	5×10^{-5}	0	0.61
2.5×10^{-5}	30	0.55	1.25×10^{-4}	0	0.25
1.25×10^{-4}	0	0.36	3.75×10^{-4}	0	0.24
			3.75×10^{-4}	30	0.05

Native invertase		
$[HgCl_2]$ mole/l	Incubation, min.	RA
10^{-6}	0	0.29
10^{-6}	30	0.29
2.5×10^{-6}	0	0.20

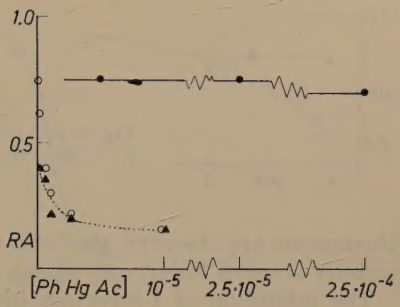

Fig. 4. Inhibition by phenyl mercury acetate (Ph-Hg-Ac): •, I-invertase; O, native enzyme, no incubation; ▲, native enzyme, 30 min. incubation.

Action of phenyl mercury acetate (Ph-Hg-Ac) on native invertase and on I-invertase.— Determinations of the relative activity (Table 4) were carried out as described in a preceding paper (11). It appears from the table and from Fig. 4 that the inhibitor has a much weaker action on I-invertase than on the native enzyme. As in the case of the inhibition by $HgCl_2$ comparatively small amounts of the inhibitors cause a partial inactivation which, however, does not increase materially even at a 100-fold inhibitor concentration. This should mean that I-invertase reacts with small amounts of the inhibitor to yield a Hg-derivative which still retains about 70 % of the activity. Similar

Table 4. Inhibition by Ph-HgAc.

pH	Ph-HgAc mole/l	Incubation, min.	RA
	I-invertase		
4.0	2.5×10^{-4}	0	0.69
5.3	6×10^{-6}	0	0.75
	2.5×10^{-5}	0	0.75
	2.5×10^{-5}	30	0.71
	2.5×10^{-4}	0	0.65
	2.5×10^{-4}	30	0.66
5.95	2.5×10^{-5}	0	0.63
	Native invertase		
4.0	5×10^{-7}	0	0.75
	5×10^{-7}	30	0.45
5.3	2.5×10^{-7}	0	0.61
	2.5×10^{-7}	30	0.41
	5×10^{-7}	0	0.45
	5×10^{-7}	30	0.36
	10^{-6}	0	0.30
	10^{-6}	30	0.21
	2.5×10^{-6}	0	0.21
	2.5×10^{-6}	30	0.20
	5×10^{-6}	0	0.18
	10^{-5}	0	0.17
	10^{-5}	30	0.17
5.95	5×10^{-7}	0	0.26
	5×10^{-7}	30	0.21

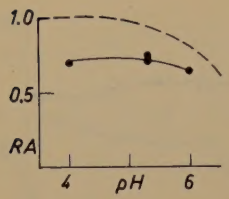

Fig. 5. pH-Dependence of the inhibition of I-invertase by Ph-Hg-Ac (- - - - activity-pH-curve).

phenomena are observed also with the native enzyme, but in this case the remaining activity is much lower, only about 20 % of the activity of the non-inhibited enzyme.

The inhibition of I-invertase by Ph-Hg-Ac does not vary appreciably with pH (Fig. 5). The same was found earlier with the native enzyme (4).

Action of o-hydroxymethylphenyl mercury chloride on invertase and I-invertase.— Table 5 shows that this very potent inhibitor of native invertase has but a weak action on I-invertase.

The experiments with $HgCl_2$ and the organic Hg compounds strongly support the assumption that, in the native enzyme, these inhibitors react essentially with HS-groups and that the action of iodine on native invertase consists in an oxidation of HS-groups, rendering them unreactive towards the Hg compounds.

If this is true, we have to assume that the HS-groups of the native enzyme are unusually stable against the action of certain oxidizing agents as ferricyanide (6) and iodoacetate (Table 6). Furthermore we have to find an explanation for the action of Ag^+ and certain other metal ions on the enzyme since, as pointed out repeatedly, the inhibition of the enzyme by these ions differs in certain respects from the Hg inhibition. Inhibition experiments with I-invertase and Ag^+ were therefore carried out.

Action of $AgNO_3$ on invertase and I-invertase.—The experiments were carried out in 0.01 M acetate buffer (9, 7, 11). Two different invertase preparations and the corresponding I-invertases were used (Table 7). Control experiments showed that the inhibition of I-invertase like that of the native enzyme is reversible, e.g. by gluta-

Table 5. Action of *o*-hydroxymethylphenyl mercury chloride at pH 5.3. No incubation.

Inhibitor mole/l	Native enzyme RA	Inhibitor mole/l	I-invertase RA
5×10^{-7}	0.76	2×10^{-4}	0.54
10^{-6}	0.55	4×10^{-4}	0.41
5×10^{-6}	0.31		
10^{-5}	0.19		

Table 6. Action of monoiodoacetate on native invertase at pH 5.3. Phosphate buffer.

$[ICH_2\text{–}COO^-]$	Incubation, 30°, min.	RA
10^{-4}	0	1.00
10^{-4}	30	0.90
10^{-3}	30	1.00
10^{-3}	30	0.90

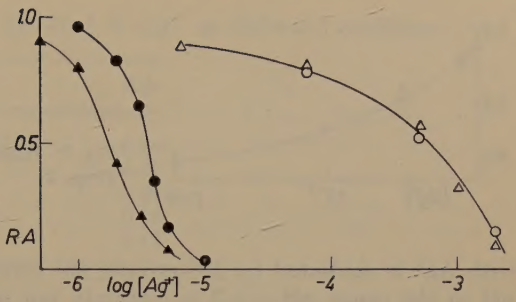

Fig. 6. Relative activity of native enzyme and I-invertase at pH 5.3 and various concentrations of Ag^+ ion. ●, native enzyme No. 1; ▲, native enzyme No. 2; ○, I-invertase No. 1; △, I-invertase No. 2.

thione, provided that enzyme and inhibitor have not been incubated together for a long time.

Table 7 and Fig. 6 (in which for obvious reasons a logarithmic abscissa has been used) show that I-invertase, compared to the native enzyme, is very little sensitive towards the Ag^+ ion. For instance, the Ag^+ concentration causing 50 % inhibition of I-invertase is more than 350 times that necessary for 50 % inhibition of the native enzyme. The conclusion appears inevitable that groups in the native enzyme which, alone or perhaps together with other groups, are largely responsible for the very high affinity of the enzyme protein for Ag^+ have been changed by the treatment with iodine. The simplest explanation would be, also in this case, that in the native enzyme the combination with Ag^+ is mediated by HS-groups and that these groups are oxidized by iodine in such a way that they lose their Ag-binding capacity. However, as pointed out before (11), it is far from evident that combination of the native enzyme

Table 7. Inhibition by Ag^+. No incubation.

Enzyme preparation 1			Enzyme preparation 2		
pH	$[Ag^+]$	RA	pH	$[Ag^+]$	RA
	Native enzyme			Native enzyme	
4.53	5×10^{-6}	0.83	5.3	5×10^{-7}	0.90
5.31	10^{-6}	0.96		10^{-6}	0.79
	2×10^{-6}	0.83		2×10^{-6}	0.42
	3×10^{-6}	0.65		3×10^{-6}	0.21
	4×10^{-6}	0.35		5×10^{-6}	0.07
	5×10^{-6}	0.17			
	10^{-5}	0.03			
	I-invertase			I-invertase	
4.8	5×10^{-6}	0.96	4.5	2×10^{-3}	0.32
5.3	5×10^{-6}	0.87	4.8	2×10^{-3}	0.19
	5×10^{-5}	0.80	5.3	5×10^{-5}	0.79
	5×10^{-4}	0.59		5×10^{-4}	0.51
	10^{-3}	0.32		2×10^{-3}	0.15
	2×10^{-3}	0.09	5.9	10^{-6}	0.93
				5×10^{-6}	0.63
				5×10^{-5}	0.51
				5×10^{-4}	0.15

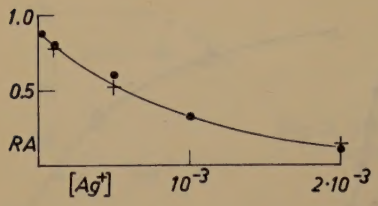

Fig. 7. Inhibition of I-invertase by Ag^+: •, Prep. 1; +, Prep. 2.

with Ag^+ is mediated by HS-groups; the strong dependence of the Ag-inhibition on pH in the range pH = 4–7 is certainly not in accord with an assumption that the reaction is simply a formation of a silver mercaptide. As shown earlier (7), the Ag-inhibition of the native invertase is explicable on the assumption that in the combination with Ag^+ groups with a pK value of about 6.7 are involved which also probably determine the alkaline branch of the activity-pH curve of the enzyme. It was also pointed out that the imidazole group of histidine has a pK value of the required magnitude, and it could be shown (6) that the combination of histidine with Ag^+ has a pH-dependence of the same type as that of the enzyme with Ag^+.

Affinity of I-invertase for Ag^+.—Fig. 7 shows the dependence of the I-invertase inhibition at pH 5.3 upon $[Ag^+]$. The Ag^+ concentrations necessary for inhibition are so large that the amount of Ag^+ bound by the enzyme (and by impurities in the enzyme preparations) is very small compared to $[Ag^+_{tot}]$. This means that the dissociation constant of the I-invertase-Ag complex is $> 10^{-4}$. This value compared to that of the native enzyme, $\sim 10^{-8}$, shows the enormous decrease of the affinity for Ag^+ caused by treatment of the enzyme with iodine.

Dependence of the I-invertase inhibition by Ag^+ upon pH.—The results of determinations at various pH values with I-invertase preparations 1 and 2 (Table 7) are shown in Fig. 8. A series with a third I-invertase preparation (Table 8) is also included. The figure shows that the Ag-inhibition of I-invertase has the same strong dependence on pH in the range pH = 4–7 as has that of the native enzyme. Since we can exclude the existence in the oxidized I-invertase of HS-groups, it seems reasonable to assume that the reaction of I-invertase with Ag^+ ions is mediated by the imidazole groups of histidine residues. It may be mentioned in this connection that the dissociation constant of the complex of free histidine with Ag^+, calculated in the same way, i.e. assuming that one histidine binds one Ag^+, is about 2×10^{-4} (7). On the other hand, the fact the the two dissociation constants have the same order of magnitude does of

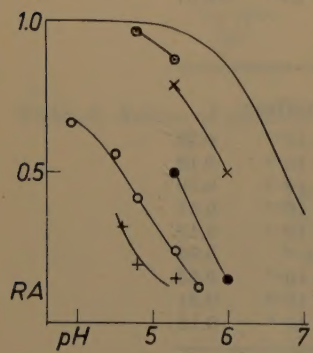

Fig. 8. pH-Dependence of the inhibition of I-invertase by Ag^+: ⊙, Enzyme preparation 1, $Ag_{tot} = 5 \times 10^{-6}$; ×, Enzyme preparation 2, $Ag_{tot} = 5 \times 10^{-5}$; •, Enzyme preparation 2, $Ag_{tot} = 5 \times 10^{-4}$; +, Enzyme preparation 2, $Ag_{tot} = 2 \times 10^{-3}$; ○, Enzyme preparation 3, $Ag_{tot} = 10^{-3}$.

Table 8. Inhibition of I-invertase by 10^{-3} N Ag^+ at different acidities.

pH	RA
3.9	0.66
4.5	0.56
4.8	0.41
5.3	0.24
5.6	0.12

Table 9. Complexing of Ag^+ by histamine. $Ag^+_{tot} = 5 \times 10^{-6}$; pH = 5.3.

[Histamine]	RA	$[Ag_{bound}]$	$[Ag_{free}]$	K
—	0.064	—	3.5×10^{-6}	—
2×10^{-3}	0.100	1.4×10^{-6}	2.1×10^{-6}	3.0×10^{-3}
4×10^{-3}	0.143	2.1×10^{-6}	1.4×10^{-6}	2.9×10^{-3}

course not prove that imidazole groups of I-invertase are responsible for the complex formation with Ag^+. It may be recalled (7) that the dissociation constant of the complex between Ag^+ and free imidazole is about 15 times as great as the corresponding constant for the histidine-Ag complex. New experiments with histamine have been carried out.

Complexing of Ag^+ by histamine.—The Ag-binding capacity of histamine was determined by measurement of the reactivation of Ag-inhibited invertase by histamine. The histamine solution was acidified to pH 5.3 before use and all experiments were carried out at this pH. Approximate values of the concentrations of histamin-Ag complex and of free Ag^+ were calculated as described earlier (7) and the same solution of native invertase was used (Table 9). The table shows that the dissociation constant of the histamine-Ag complex is about 3×10^{-3}, i.e. it has the same order of magnitude as the dissociation constant of the imidazole-Ag complex.

Action of copper(II) ions on I-invertase and native invertase.—Experiments with invertase and copper(II) ions give considerably less reproducible values than those with Ag^+. Incubation of the enzyme with Cu^{++} ions causes fairly rapid increase of the inhibiting action, and the inhibition in experiments with incubation of enzyme and Cu^{++} is only partly reversible, e.g. by H_2S. A reasonable explanation would be that the copper ions catalyze oxidative destruction of the enzyme.

The following experiments were carried out without incubation of enzyme and inhibitor, i.e. the enzyme was added last to a mixture of substrate, $CuSO_4$ solution, acetate buffer (0.01 *M*) and water and the relative activity was determined immediately. Table 10 shows that I-invertase is considerably less inhibited by Cu^{++} than is the native enzyme.

Action of Cd^{++} on I-invertase and native invertase.—The same method was used as in the case of the inhibition by Cu^{++}. It appears from Table 11 that the action of Cd^{++} on I-invertase is much weaker than the action on the native enzyme.

Since the inhibition of invertase by Cu^{++} and Cd^{++} shows the same characteristic dependence on pH as the inhibition by Ag it has been concluded (4) that the combina-

Table 10. Action of Cu^{++} on native invertase and on I-invertase.

Native invertase			I-invertase		
pH	$[Cu^{++}]$	RA	pH	$[Cu^{++}]$	RA
5.3	5×10^{-4}	0.18	5.3	5×10^{-4}	0.57
5.95	5×10^{-5}	0.17	5.3	5×10^{-3}	0.22

Table 11. Inhibiting action of the Cd^{++} ion.

Native enzyme			I-invertase		
pH	$[Cd^{++}]$	RA	pH	$[Cd^{++}]$	RA
5.30	5×10^{-4}	0.82	5.95	5×10^{-3}	0.52
	2.5×10^{-3}	0.66		10^{-2}	0.27
	5×10^{-3}	0.11			
5.95	2×10^{-4}	0.36			
	10^{-3}	0.13			

tion of invertase with Ag^{+}, Cu^{++} and Cd^{++} (and with Pb^{++}, Zn^{++} etc.) is mediated by the same group(s) of the enzyme protein. The results on the inhibition of I-invertase by the metal ions mentioned is in accordance with this view.

Discussion

The experiments on the action of iodine on invertase and on the inhibition of I-invertase by the metal ions investigated lead to the following tentative explanation of the results.

The action of iodine is best explained as an oxidation of HS-groups of the enzyme molecule, resulting either in the formation of disulphidic bonds, or in an "overoxidation" of HS-groups, e.g. to sulphinic acid groups or still higher oxidation levels. Anyway, treatment of the oxidized product with a number of reducing agents, calculated to reduce –S–S- to HS-bonds, have not been found to reactivate the I-invertase.

The *inhibition of I-invertase* by Ag^{+}(Cu^{++}, Cd^{++}, etc.) ions may be explained as an interaction of the metal ions with imidazole groups of histidine residues in the oxidized enzyme. The dissociation constant of the Ag-complex seems to have the right order of magnitude, and the dependence on pH of the inhibition of I-invertase by the metal ions mentioned supports the view that the imidazole group but not its protonated form binds the metal ions. The "acid" group of invertase (to use Michaelis' designation) is supposed to have p$K \sim 6.7$; the pK values of the imidazole of histidine derivatives have approximately the same magnitude.

This explanation of the action of Ag^{+} etc. on I-invertase is in good agreement with earlier investigations on the binding of metal ions, especially Zn^{++} ions, to proteins and imidazole derivatives (12).

The weak inhibiting action of $HgCl_2$ and certain organic mercury compounds upon I-invertase does not show the same strong dependence on pH as that of Ag^{+} etc. One explanation of the action of the Hg compounds would be, that certain HS-groups of the native enzyme remain in this form in I-invertase and react with the

inhibitors. However, the action of the Hg compounds on I-invertase is so weak that this explanation does not appear probable and there are certainly several other groups in the oxidized protein that may react with Hg compounds.

If it is assumed that the action of iodine on invertase is an oxidation of HS-groups of the protein, then it remains to explain the very strong *inhibition of the native enzyme* by the metal inhibitors and its dependence on pH. There seems to be no difficulty in explaining the inhibition by the Hg compounds; their strong inhibiting action at very low inhibitor concentrations, and the fact that their action does not vary appreciably with pH may be explained as the wellknown reaction between HS-groups and Hg compounds. However, it should be remembered that the inhibition by these compounds is competitive, i.e. that substrate-binding groups are involved in the combination with Hg. From reasons that will be explained in the following it appears probable that the HS-groups do not participate in the attachment of substrate molecules to the enzyme surface. The formation of the Hg compounds of the enzyme must therefore be supposed to influence also other groups than the sulphydryl groups.

If it be supposed that heavy metal ions in general which inhibit the enzyme, do this by way of interaction with HS-groups, then one asks oneself why the inhibition of the enzyme by Ag^+ (Cu^{++} etc.) depends so strongly on pH that, e.g., an amount of Ag^+ giving 100 % inhibition at pH 7 has practically no inhibiting action at pH 4. One is forced to assume that groups in the enzyme protein which change their charge nearly completely in the pH-range mentioned are involved in the combintion with the metal ions. But if we assume that these groups are imidazole groups (or other groups with about the same pK-values), then it is hard to understand why the metal ions should preferably react with these groups and not with the HS-groups which in all probability must have a much higher affinity for the metal ions.

What is known at present about the interaction of heavy metal ions with yeast invertase seems to be best explained by the assumption that the inhibitors act, in principle, in the generally accepted way on HS-groups of the enzyme protein but that these groups in the active enzyme, for sterical reasons, i.e. the occurrence of groups of such nature and in such positions that an interaction with the HS-groups is probable, are strongly modified in their chemical behaviour. Obviously, this interaction must be supposed to be strongly dependent on the pH. The groups interacting with the HS-groups must have pK-values around 6.7 and, as pointed out before, it seems reasonable to assume that they are imidazole groups of histidine residues. Then we have to conclude that interaction with HS is possible in the protonated imidazole groups but not in the neutral ones as shown in Fig. 9, in which the the interaction has been represented schematically by a hydrogen bond S–H–N.

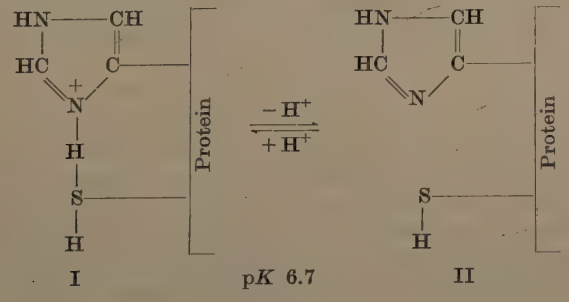

Fig. 9.

It is true that generally the tendency of sulphur to participate in hydrogen bonds as assumed in Fig. 9 is not very accentuated. However, it does not appear impossible that bonding of this type could have a considerable stability under unusually favourable sterical conditions. Interactions of a similar type between HS-groups and certain other groups have been assumed earlier (13) in order to explain the variable reactivity of sulphydryl groups in peptides.

In the case of invertase, the assumption of stabilizing hydrogen bonds may give an explanation of the fact that the enzyme, in spite of its nature of a sulphydryl enzyme, shows a marked stability against certain oxidants as O_2 and ferricyanide, and against alkylating agents as iodoacetate.

It should be emphasized that Fig. 9 is not meant to be taken literally. It seems probable that a hydrogen bond like that tentatively assumed would, even if the participating groups were very favourably situated, be too weak to explain the non-reactivity of the HS-groups toward Ag^+. It could be that the loss of the proton with $pK \sim 6.7$ is accompanied by a more radical, reversible change ("denaturation") of the protein structure in the neighbourhood of the groups involved. In the active ("native") form of the enzyme the HS-groups may be "masked" to an extent that prevents, i.a., their reaction with silver ions, not merely by an interaction with imidazole but as a consequence of the "tertiary" protein structure as a whole. The structural change (reversible denaturation) brought about by the removal of the proton would then "unmask" the HS-groups making them available to the metal ions.

The loss of the proton, followed by a more or less radical change of protein structure, transforms the enzyme reversibly to an enzymatically inactive form; this is the explanation of the alkaline branch of the activity-pH-curve. The presence of Ag^+ or certain other metal ions reacting with the "denatured" form of the enzyme will displace the equilibrium between the active and the inactive forms, i.e. cause an inhibition dependent on pH.

Since the inhibition of the native enzyme by Hg compounds is rather independent of pH we have to assume that these, owing to their very high affinity to sulphydryl groups, react even with the more or less "masked" HS-groups (I in Fig. 9); that they react with free HS-groups (II in Fig. 9) is a matter of course. Earlier experiments with highly purified yeast invertase (4) appear to intimate that the inhibiting action of $HgCl_2$ increases somewhat towards the alkaline region of the pH-optimum; this may suggest that the free HS-groups react with Hg somewhat easier than the "masked" groups. It should also be remembered that the inhibition of the enzyme by Hg compounds increases if enzyme and inhibitor are incubated together before the determination of RA.

The inhibition by $HgCl_2$ is competitive; since the inhibition by Ag^+ is non-competitive it may well be that in the combination of the enzyme protein with Hg also other groups than the sulphydryl groups are instrumental.

The action of Ag^+ (Cu^{++} etc.) ions on the native invertase shows that groups of the protein with $pK \sim 6.7$ are involved. The imidazole group of histidine has a pK-value of this magnitude, and only neutral imidazole (not the protonated form) binds Ag^+. However, the affinity of histidine for Ag^+ is about 10^4 times lower than that of the native enzyme for the silver ion. Thus it is not possible to assume that imidazole groups alone are responsible for the combination of the enzyme with Ag^+. However if it is assumed that the protonated imidazole but not the neutral form interacts with a suitably situated HS-group (Fig. 9) then the pK-value of the imidazole

group may be reflected in the reactivity of the HS-group: In acid solution the protonated imidazole group prevents the reaction between the HS-group and Ag^+, whereas in neutral or alkaline solution no interaction exists, and the HS-group shows its more or less normal capacity for attachment of the silver (copper etc.) ions. Thus the HS-groups of native invertase, in their behaviour towards certain metal ions and certain other sulphydryl reagents, will seemingly exhibit pK-values of quite unexpected magnitude.

As mentioned before it has been assumed that the group(s) in the invertase molecule reaction with Ag^+ is the "acidic" group which, according to Michaelis, determines the alkaline branch of the activity-pH-curve of the enzyme (14). In the language of today, enzyme activity is abolished if this group has lost its proton. If the explanation of the metal ion inhibition of yeast invertase given above is correct, we have to conclude that the "dissociation" of the acid group, postulated by Michaelis, is governing the equilibrium, depicted in Fig. 9. This must, of course, not necessarily mean that the existence of the assumed hydrogen bond (I in Fig. 9) is necessary for enzyme activity. It may be that the positively charged imidazole group is sufficient, irrespective of an eventual interaction with the HS-group. Actually, since the oxidized enzyme, I-invertase, has a well defined enzymatic activity, it does not appear probable that the assumed interaction should be a prerequisite for activity. It is true that the state of the S atoms in I-invertase is not known; however the existense of a comparable interaction between imidazole and the sulphur of I-invertase appears improbable.

In this connection it seems appropriate to repeat that the affinity for the substrate and the dependence of the activity on pH is the same for the native enzyme and for I-invertase; this is in accord with the assumption that the activity-pH-curves are determined, in both cases, by the pK-value of imidazole groups which are not involved in the combination of the enzymes with their substrates.

The equilibrium I $\rightleftharpoons$ II (Fig. 9) is not dependent upon the substrate concentration. Kuhn (15) found that the alkaline branch of the activity-pH-curve of yeast invertase is independent of the saccharose concentration in the assay mixtures, and the explanation is (4, 16) that the group(s) which determine this part of the activity-pH-curve are not involved in the attachment of the substrate to the enzyme. The fact that the inhibition of invertase by Ag^+ (Cu^{++} etc.) is non-competitive is quite in agreement with the assumption that the alkaline branch of the activity-pH-curve and the binding of the metal ions are determined by the same group(s). The structures I and II (Fig. 9) and the Ag compound of II are equally well capable of combining with saccharose, but only the enzyme-substrate-compound having structure I is active, i.e. desintegrates into enzyme and reactions products.

The authors want to express their gratitude to professor Paul D. Boyer, University of Minnesota, for a discussion of certain problems in connexion with this investigation.

SUMMARY

Treatment of yeast invertase with iodine results in the formation of a derivative, I-invertase, having a well defined enzymatic activity = 55 % of that of the native enzyme. Experiments on the inhibition of the native enzyme and of I-invertase by heavy metal ions and organic mercury compounds show that the native enzyme is strongly inhibited by extremely low concentrations of the inhibitors whereas I-invertase is much less affected. The action of iodine on the native enzyme is explained as an oxidation of sulphydryl groups of the enzyme protein. The inhibition

of native enzyme (and I-invertase) by Ag^+ and certain other metal ions shows a characteristic dependence on pH, intimating that in the combination of enzyme protein and metal, groups of the protein are involved which have a pK-value ~ 6.7. Various facts appear to show that these groups may be identified with imidazole groups of histidine. However, the strong inhibition of the native enzyme by very low concentrations of Ag^+ cannot be explained on the assumption that imidazole groups alone are responsible for the binding of the metal ion. The fact that I-invertase is very little affected by Ag^+ clearly shows that HS-groups of the native enzyme are the principal points of attachment of the metal ion. As an explanation of these seemingly contradictory results it has been assumed that in the native enzyme HS- and imidazole groups are in such sterical positions that a fairly strong interaction exists between the SH-group and the protonated (but not the neutral) imidazole group.

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On the complex formation between silver and thiosulphate ions

I. Solubility measurements

By Rolf O. Nilsson

With 5 figures in the text

The complex formation between silver and thiosulphate ions has been the subject of much interest on account of its great practical application in the photographic fixing process and this interest has resulted in the synthesis of many salts containing silver, thiosulphate, and some alkali or alkaline earth metal. Careful investigations by Baines [1, 2] and Basset and Lemon [3], have shown that the only solid salts which exist in the sodium thiosulphate—silver thiosulphate— water system (the only system dealt with here) are the ones given in Table 1. As the above authors have reviewed the literature concerning the syntheses of such salts and as since then no new salts have been prepared, other authors are not cited here. With the aid of physico-chemical methods complex ions in *solutions* have been studied by several authors ([4] to [28]) and the results of their measurements are collected in Table 1 in chronological order. All the methods used are not of the same usefulness and the ionic strength has not in most cases been kept constant, but in spite of this many investigators agree about the existence of the ion $Ag(S_2O_3)_2^{3-}$ in a medium range of thiosulphate concentrations. $AgS_2O_3^-$ has been proposed to exist at low concentrations (its complexity constant has been given by Chateau and Pouradier [22, 26]) and at high concentrations the existence of $Ag(S_2O_3)_3^{5-}$ has been proved [4, 15, 22]. The suggestions made by different authors concerning the polynuclear groups do not, however, show much agreement and consequently a reinvestigation of the system was still of interest.

In this paper the results of solubility measurements are dealt with and in a following paper a potentiometric investigation will be reported (Nilsson [28]). Under both investigations $NaClO_4$ was added to an ionic strength $4\,M$. As can be seen from Table 1, the polynuclear complexes proposed by the present author, are not the same as the ones suggested by earlier investigators.

Method

In the present investigation the solubility of $NaAgA \cdot H_2O$ in sodium thiosulphate solutions of different concentrations, $0 \leqslant c_{Na_2A} \leqslant 0.267\ M$ (where A stands for S_2O_3), was measured at 25°C and the solubility data was handled according to a method

Table 1. Work on complex formation between silver and thiosulphate ions. The complexity constants, $\beta_{m,n}$, are defined by equation (10). S_2O_3 is abbreviated as A.

Author	Method [1]	Ionic medium	°C	$\log \beta_{1,1}$	$\log \beta_{1,2}$	$\log \beta_{1,3}$	Remarks
Baines [1, 2]							Solid salts: $NaAgA \cdot H_2O$, $Na_5Ag_3A_4 \cdot 2H_2O$
Basset and Lemon [3]			25				Solid salts: $NaAgA \cdot H_2O$, $Na_3AgA_2 \cdot 2H_2O$, Ag_2A, $NaAg_3A_2 \cdot H_2O$, $Na_5Ag_3A_4 \cdot 2H_2O$
Bodländer [4]	P	Varying			12.99	13.54	
Slator [5]	R		25	Ev. [2]	Ev.		
Müller [6]	P	Varying	1 and 18	Ev.			Suggests $Ag_4A_3^{2-}$ and Ag_2A
Pawelka [7]	P				13.88		
Carrière and Raulet [8]	S_1, C		20	Ev.			From their measurements $\log \beta_{1,1} = 8.87$ can be calculated [22].
Spacu and Murgulescu [9]	P	Varying	0	Ev.	Ev.	Ev.	Suggest $Ag_4A_3^{2-}$ and $Ag_2A_3^{4-}$
Masaki [10]	P	Varying	25		13.73		
Ferrell et al. [11]	P	Varying	Room temp.		13.30	Ev.	
van Rysselberghe and Knapp [12]	T	0.1 *M* Na_2A, 0.08 *M* AgCl	25	Ev.			
Brintzinger and Eckardt [13]	D	Varying	25				Suggest $Ag_2A_2^{2-}$ and $Ag_2A_6^{10-}$
Schmitz-Dumont and Schmitz [14]	P	2 *M* Na_2A	0 and 16			Ev.	
Ölander and Adelsohn [15]	P	2 *M* $NaClO_4$	25		12.78	13.06	Suggest $Ag_2A_2^{2-}$ and $Ag_2A_3^{4-}$
Dey and Mushran [16]	Cg						Suggest a bivalent ion ($Ag_2A_2^{2-}$?)
Dei [17]	S_1		20, 30 and 75	Ev.	Ev.		
Brehmer [18]	P	Varying	17		13.19		
			20.8		13.16		
			29.9		12.80		
Uryu [19]	P, F	Varying				Ev.	

Chateau and Pouradier [22]	P	→0	20	8.83	13.46	14.15	$\log \beta_{AgIA} = 14.57$
Faucherre [23]	P	1 *M* $NaClO_4$			Ev.		
Jaenicke [24]	P	0.2 *M* $NaNO_3$	20	10	14.36		
Saxena and Jain [25]	C			Ev.	Ev.		Suggest $Ag_3A_4^{5-}$ and Ag_2A
Chateau and Pouradier [26]	P	→0	70	7.45	11.60		
Chateau, Hervier, and Pouradier [27]	P	→0	25		13.46		
			35		13.02		
			49.5		12.43		
			68		11.68		
This work	S_2	4 *M* $NaClO_4$	25	7.36	12.72		Evidence for the series Ag_mA_{m+2} ($m \geq 1$)
							$L = [Na^+]\,[Ag^+]\,[A^{2-}] = 2.28 \cdot 10^{-13}$ in solns. saturated with $NaAgA \cdot H_2O$
Nilsson [28]	P	4 *M* $NaClO_4$	25		12.72	13.52	Polynuclear complexes of the composition Ag_mA_{m+2} were found

[1] C = conductometric measurements, Cg = coagulatinn experiments, D = dialysis, F = freezing point depression, P = potentiometric measurements, R = reaction velocity measurements, S_1 = solubility of AgX (X = halogen) in Na_2A, S_2 = solubility of $NaAgA \cdot H_2O$ in Na_2A.

[2] Evidence for AgA^-.

outlined by Leden [29]. The "ionic strength" in the solutions to be saturated, was kept constant at 4 *M* by adding sodium perchlorate. Equation (1) gives the relation between the concentrations of sodium perchlorate and sodium thiosulphate

$$c_{NaClO_4} + 3c_{Na_3A} = 4. \tag{1}$$

The sodium ion concentration is given by the equation

$$[Na^+] = 4 + l - c_{Na_3A} \tag{2}$$

where l is the solubility of $NaAgA \cdot H_2O$.

From Table 2 can be seen that the sodium ion concentration varies with at most 2.3 % in the saturated solutions, which is favourable as the complexes formed are negatively charged, and the concentrations of positive ions therefore should be kept as constant as possible. The dissolution of the salt can be written as

$$m\ NaAgA \cdot H_2O_{(s)} + x\ A^{2-} \rightleftarrows Ag_m\ A_{m+x}^{(m+2x)-} + m\ Na^+ + m\ H_2O \tag{3}$$

and as $[Na^+]$ and $[H_2O]$ are approximately constant the system is defined by the equilibrium constants

$$k_{m,x} = \frac{[Ag_m\ A_{m+x}]}{[A]^x}. \tag{4}$$

The solubility is now given by

$$l = \sum_{m=1} \sum_{x=-m} m\,[Ag_m\ A_{m+x}] = \sum\sum m\,k_{m,x}\,[A]^x = \sum_{x=-m} K_x\,[A]^x \tag{5}$$

where consequently

$$K_x = k_{1,x} + 2\,k_{2,x} + \cdots = \sum_{m=1} m\,k_{m,x}. \tag{6}$$

The total thiosulphate content is

$$c_A = [A] + \sum_{m=1} \sum_{x=1-m} (m+x)\,[Ag_m\ A_{m+x}] = [A] + \sum\sum (m+x)\,k_{m,x}\,[A]^x. \tag{7}$$

The average number of thiosulphate groups bound to each silver atom is defined by

$$\bar{n} = \frac{c_A - [A]}{l} = \frac{\sum_{m=1} \sum_{x=1-m} (m+x)\,k_{m,x}\,[A]^x}{\sum_{x=-m} K_x\,[A]^x} \tag{8}$$

and the amount of the silver which is bound as $Ag_m\ A_{m+x}$ is

$$\alpha_{m,n} = \frac{m\,[Ag_m\ A_{m+x}]}{l} = \frac{m\,k_{m,x}\,[A]^x}{\sum K_x\,[A]^x}. \tag{9}$$

Between the complexity constants

$$\beta_{m,n} = \frac{[Ag_m\ A_n]}{[Ag^+]^m\,[A]^n} \tag{10}$$

and the $k_{m,x}$ constants the following relation exists

$$k_{m,x} = k_{m,n-m} = (k_{1,-1})^m \beta_{m,n}. \tag{11}$$

From equation (5) follows that the constants K_x, but not the individual constants $k_{m,x}$, can be determined in the series, if l is known as a function of [A]. The evaluation of [A] and of the constants K_x will be described in a following section.

To determine the product $k_{1,-1} = [Ag^+]\,[A]$, which is a constant if $[Na^+]$ and $[H_2O]$ are constant, the concentration of the free silver ions was measured with the aid of silver electrodes in the saturated solutions. The following cell was used.

$$-\,Ag \left| \begin{matrix} \text{saturated} \\ \text{solution} \end{matrix} \right| 4\ M\ NaClO_4 \,|\, RE+. \tag{12}$$

The emf of cell (12) is

$$E_m = E_0 - 59.16 \log[Ag^+] = -85.7 - 59.16 \log[Ag^+]. \tag{13}$$

The value of E_0 was determined by taking readings on the cell given in (12) when the saturated solution was exchanged with silver perchlorate solutions of different concentrations. It was thereby also proved that the electrodes followed Nernst's law when $c_{Ag} > 1$ mM. No corrections for the diffusion potentials were made as the ionic mobilities of the complexes formed were not known. The experimental values are collected in Table 2.

Experimental

Reagents

4 M $NaClO_4$ was made as previously described (Nilsson [30]). *4/3 M Na_2A* was made from $Na_2A \cdot 5H_2O$ (Merck's pro analysi quality). The concentration was determined iodometrically.

$NaAgA \cdot H_2O_{(s)}$ was prepared according to Baines [1]. The product contained 3.819 mmol Ag per gram (electrolytically) and 3.834 mmol A (iodometrically). The electronegativity requires 3.849 mmol Na and the rest, 88.5 mg, corresponds to 3.862 mmol H_2O. X-ray powder photographs were taken of the crystals using a Guinier camera.

The *silver electrodes* were made by electrolytic precipitation of silver from a silver cyanide solution on platinum wires according to Brown [31] although thicker platinum wires were used ($\theta \approx 1.2$ mm).

The *potentiometer*, the *galvanometer* and the *reference electrodes* used were the same as described in Ref. [30] and the *nitrogen* was purified as described there.

Procedure

The $NaAgA \cdot H_2O$ crystals were transferred to glass tubes containing solutions with the compositions given by eq. (1) where $0 \leqslant c_{Na_2A} \leqslant 0.267\ M$. The tubes were then rubberstopped and rotated 6–18 hours in a waterthermostat at 25.00 ± 0.02°C. In the solutions with $c_{Na_2A} \geqslant 75$ mM a slow decomposition set in after 18 hours when a gelatinous precipitate was formed. To check if this decomposition had any influence on the solubility, crystals were rotated in a solution with $c_{Na_2A} = 0.267\ M$ and the solubility was determined after 0.5, 1, 2, and 18 hours (see Table 2). It was found that

Table 2. The solubility, l, of $NaAgA \cdot H_2O$ in sodium thiosulphate solutions at 25°C. Ionic strength $\approx 4\ M$. $k_{1,-1} = 5.7 \cdot 10^{-14}$, $k_{1,0} = 1.3 \cdot 10^{-6}$, $k_{1,1} = 0.30$, and $K_2 = 8$. The numbers in parentheses in the table head refer to equation numbers.

c_{Na_2A} mM	Time of rotation h	Method of analysis[1]	$l_{exp.}$ mM	$[Na^+]$ M (2)	$[Ag^+]$ 10^{13} M (13)	[A] mM (15)	[A] mM (18)	$\bar{n}$ exp.	$\bar{n}$ calc. (8)	$l_{calc.}$[2] mM	$l_{calc.}$[3] mM
0	46	R	0.00157	4.00			0.000209		0.87	0.00163	
0.002	46	R	0.0021	4.00			0.00157		1.23	0.00181	
0.005	46	R	0.0022	4.00			0.00386		1.47	0.00247	
0.010	37	R	0.00334	4.00			0.00770		1.64	0.00362	
0.020	37	R	0.00696	4.00	(31 200)	(0.0184)	0.0154		1.78	0.00592	
0.050	36	R	0.0142	4.00	(14 900)	(0.0386)	0.0385	(1.82)	1.90	0.0129	
0.100	36	R	0.0240	4.00	7 500	0.076		2.00	1.94	0.0242	
0.200	36	R	0.0502	4.00	3 760	0.152		1.96	1.98	0.0471	
0.500	36	R	0.116	4.00	1 520	0.375		2.08	1.99	0.115	
1	36	R	0.234	4.00	762	0.748		2.08	2.00	0.229	
2	18	R	0.460	4.00	382	1.49		2.11	2.00	0.466	
5	18	R	1.29	4.00	155	3.68		2.00	2.00	1.21	
5	16	E_d	1.27	4.00	149	3.83		1.92	2.00	1.27	
10	16	R	2.64	3.99	79.6	7.17		2.07	2.00	2.56	
10	12	E_d	2.59	3.99	75.5	7.54		1.95	2.00	2.71	
15	16	E_d	3.98	3.99	51.8	11.0		2.01	2.00	4.27	
20	16	E_d	5.78	3.99	39.8	14.4		1.97	2.00	5.98	
35	16	E_d	12.1	3.98	24.8	23.1		1.98	2.00	11.2	11.3
50	16	E_d	17.3	3.97	18.2	31.6		2.07	2.00	17.5	18.0
65	16	E_d	23.8	3.96	15.0	38.4		2.12	2.01	23.3	24.7
75	12	E_d	30.2	3.96	14.0	41.2		2.12	2.01	25.9	27.9
75	21	E_d	30.3	3.96	13.7	42.1		2.08	2.01	26.7	28.9
108	16	E_d	49.0	3.95	10.6	54.4		2.09	2.01	40.0	48.1
175	18	E_d	97.0	3.92	8.10	71.9		2.06	2.01	63.1	95.9
200	18	E_d	116.8	3.92	7.64	76.2		2.06	2.01	69.3	113.0
267	18	E_d	174.9	3.91	6.67	87.6		2.02	2.01	86.9	174.6
267	0.5	E_d	161.9								
267	1	E_d	172.5								
267	2	E_d	174.9		6.30	93.0		2.00			

[1] E_d = electrochemical deposition of Ag, R = radioactive tracer technique.
[2] calculated acc. to (19) with the constants in the table head.
[3] calculated acc. to (19) with the constants in the table head and in addition to them $K_5 = 17000$.

the solubility after two hours, when no decomposition was visible, was the same as after 18 hours, when decomposition had set in, while the measured value for $[Ag^+]$ was about 5 % lower than after 18 hours. The values of l and $[Ag^+]$ for the solutions where $c_{Na_2A} \geq 75$ mM may consequently be regarded as fairly true in spite of the slight decomposition observed. The solutions were filtered under N_2-pressure through colloid filters in a thermostatically controlled Thiessen apparatus. The silver contents in those saturated solutions where $c_{Na_2A} \geq 5$ mM were determined electrolytically from a cyanide bath at 3 V and 0.1 A. In the solutions with $c_{Na_2A} \leq 10$ mM crystals containing some radioactive ^{110}Ag were used. These crystals darkened on standing after a few weeks when kept dry. When the saturated solutions were filtered the first

10 ml of the filtrates were discarded to avoid errors from adsorption in the filter apparatus. The rest of the filtrate was divided into 10 ml portions to which some NaCN and $AgNO_3$ was added (to avoid adsorption on standing) and the radioactivity of the portions was measured in an alcoholquenched liquid counter tube (GM 6). The specific activity was determined by measuring the radio activity of part of the solution with known silver contents, from which the crystals were precipitated, after diluting it with 4 M $NaClO_4$. At the lowest solubilities 60 cpm were measured from which 30 cpm were due to cosmic radiation. The relative error was consequently rather large here, but it diminished rapidly with increasing solubility. At $c_{Na_2A} = 5$ and 10 mM the solubility was determined both by the radioactive method and electrolytically, and the results obtained by the two methods agreed to within two per cent. X-ray photographs, taken of the crystals remaining in the saturated solutions showed $NaAgA \cdot H_2O$ to be the only solid phase present.

To extend the range of measurements it was also tried to dissolve radioactive $NaAgA \cdot H_2O$ in solutions containing $2 \cdot 10^{-6}$ and $4 \cdot 10^{-6}$ M radioactive $AgClO_4$. It was, however, found that the radioactivities of the saturated solutions in these cases were the same as if no silver perchlorate had been present from the beginning. This effect is probably due to an adsorption of the silver ions on the crystals as adsorption on glass under similar conditions was not found to be the case. $NaAgA \cdot H_2O$ was also tried to be dissolved in 10 mM $AgClO_4$, but the crystals darkened in 10 minutes, probably due to sulphide formation. It is a well-known fact that precipitated Ag_2A in the presence of silver ions decomposes almost immediately under formation of Ag_2S and it is possible, that the above-mentioned adsorption of silver ions is the first step in a process which leads to formation of Ag_2S.

Nitrogen was bubbled through the saturated solutions during the measurements in cell (12). The emfs could be repoduced within 0.5 mV. The calculated $[Ag^+]$ values are also impaired by an error coming from the unknown diffusion potentials for which no corrections could be made.

Evaluation of the equilibrium constants

In Table 2 the measured quantities l and $[Ag^+]$ are given as functions of c_{Na_2A}. For determination of K_x (5) one must know l as a function of [A] and this quantity was therefore evaluated in the following way: log l was first drawn against log c_{Na_2A} and this curve had in a wide range of concentrations a slope ≈ 1. As the AgA_2 ion would give the slope ≈ 1, it was assumed that $\bar{n}$ had the value of 2 in this range. [A] was now calculated according to

$$[A] = c_A - \bar{n} \cdot l = c_{Na_2A} - (\bar{n} - 1)l \qquad (14)$$

and the product $k_{1,-1} = [Ag^+][A]$ could then be obtained. It turned out to be practically constant ($k_{1,-1} = 5.7 \cdot 10^{-14}$) in a wide range of concentrations, $0.025 \leqslant l \leqslant 5$ mM. As in this range $[Na^+] = 4\ M$ the product

$$L = [Na^+][Ag^+][A] \qquad (15)$$

becomes $L = 4 \cdot 5.7 \cdot 10^{-14} \approx 2.3 \cdot 10^{-13}$. The constant value of $k_{1,-1}$ shows that the assumed value $\bar{n} = 2$ is correct. Not only AgA_2^{3-}, but also every other complex with the composition Ag_mA_{m+1} would give a straight line with the slope ≈ 1 in a log l *vs.*

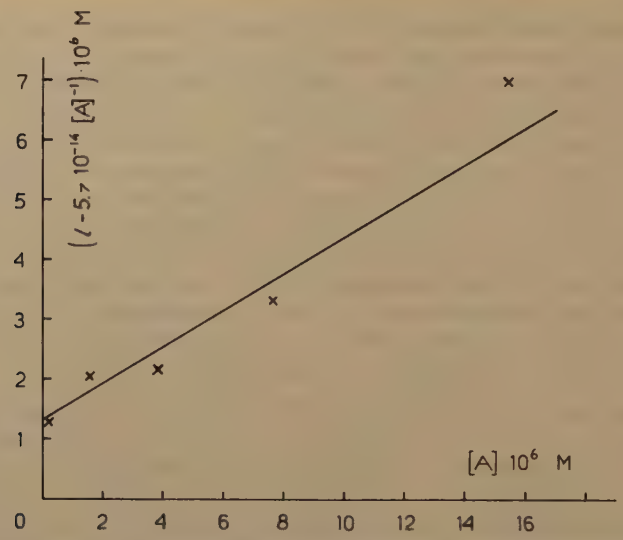

Fig. 1. $l - k_{1,-1}[A]^{-1}$ *vs.* [A]. The origo ordinate gives $k_{1,0} = 1.3 \cdot 10^{-6}$ (*cf.* eq. (19)). The drawn curve is calculated with the constants $k_{1,0} = 1.3 \cdot 10^{-6}$, $k_{1,1} = 0.30$, and $K_2 = 8$.

log c_{Na_2A}-diagram, but if one or more complexes with $m > 1$ had been present they would have given rise to $\bar{n} < 2$ and one should accordingly not have got a constant value of $k_{1,-1}$ in the operation described. It has moreover been proved by potentiometric measurements in unsaturated and in supersaturated solutions (Nilsson [28]) that the only polynuclear complexes which are formed in considerable amounts, all belong to the series Ag_mA_{m+2} and their contributions to the solubility is consequently included in the term $K_2[A]^2$ (*cf.* equations 3, 4, 6, and 5). The other constants accordingly represent mononuclear complexes i.e. $K_0 = k_{1,0}$ and $K_1 = k_{1,1}$. When $k_{1,-1}$ and L had been determined in the way described, the value of [A] could be calculated from (15) in the range where $[Ag^+]$ could be measured, i.e. where $l \geqslant 0.05$ mM. In the solutions where $l \leqslant 0.025$ mM, $[Ag^+]$ could not be measured on account of the low silver contents, and [A] was therefore in this range calculated according to (18), which is obtained by subtracting (17) from (16).

$$c_A = [A] + k_{1,0} + 2\,k_{1,1}[A] \tag{16}$$

$$l = k_{1,-1}[A]^{-1} + k_{1,0} + k_{1,1}[A] \tag{17}$$

$$c_A - l = [A] - k_{1,-1}[A]^{-1} + k_{1,1}[A] \tag{18}$$

The complexes which are represented by the term $K_2[A]^2$ make in the actual range less than 1 % of the solubility and can therefore be excluded in (16), (17), and (18). In calculating [A] from (18) a preliminary value of $k_{1,1}$ was used, which was obtained as the slope of the curve in a $(l - k_{1,-1}[A]^{-1})$ *vs.* [A] diagram in the range where $0.025 \leqslant l \leqslant 5$ mM.

We now know l as a function of [A] through the whole range of concentrations and can determine the constants in the power series,

$$l = k_{1,-1}[A]^{-1} + k_{1,0} + k_{1,1}[A] + K_2[A]^2 + K_3[A]^3 + \cdots. \tag{19}$$

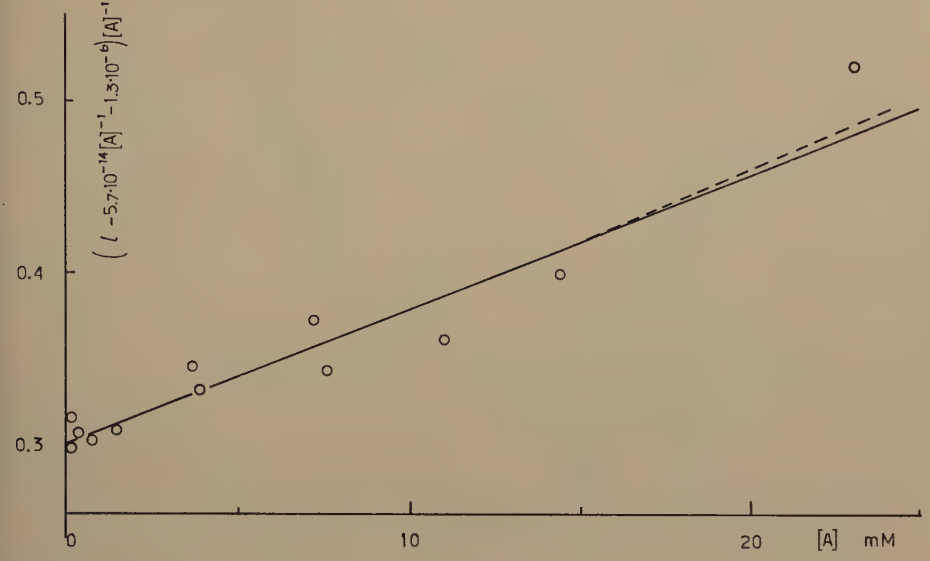

Fig. 2. $(l - k_{1,-1}[A]^{-1} - k_{1,0})[A]^{-1}$ *vs.* [A]. The origo ordinate gives $k_{1,1} = 0.30$ (*cf.* (19)). The drawn curve is calculated with $k_{1,1} = 0.30$ and $K_2 = 8$ and the dotted curve with $k_{1,1} = 0.30$, $K_2 = 8$, and $K_5 = 17000$.

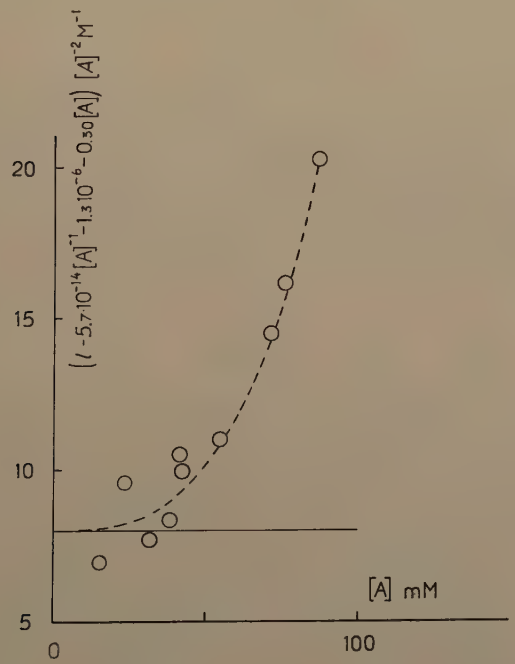

Fig. 3. $(l - k_{1,-1}[A]^{-1} - k_{1,0} - k_{1,1}[A])[A]^{-2}$ *vs.* [A]. The dotted curve calculated with $K_2 = 8$ and $K_5 = 17000$, and the horizontal line with $K_2 = 8$ and $K_x = 0$ for $x > 2$.

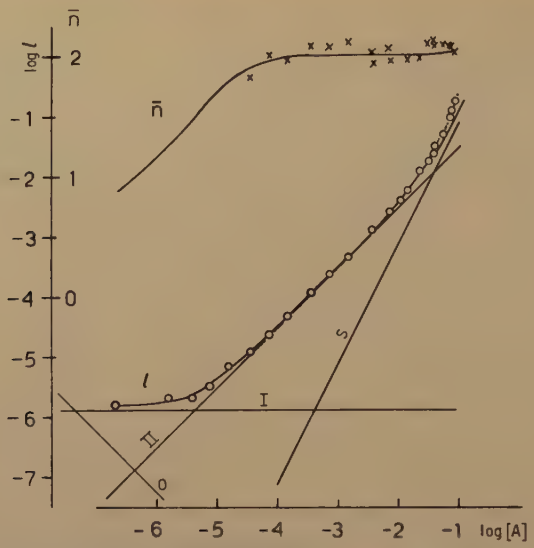

Fig. 4. log l (ooo) and $\bar{n}$ (xxx) *vs.* log [A]. The drawn solubility curve is calculated according to (19) with the constnats $k_{1,-1} = 5.7 \cdot 10^{-14}$, $k_{1,0} = 1.3 \cdot 10^{-6}$, $k_{1,1} = 0.30$, and $K_2 = 8$. The $\bar{n}$-curve is calculated with the same constants according to (8). The dotted solubility curve is calculated with the constants given above and in addition to them $K_5 = 17000$. The concentration of the species Ag^+, AgA, and AgA_2 at a certain value of [A] can be directly read off from the lines marked 0, I and II respectively. The line marked S gives $K_2[A]^2 = \Sigma m[Ag_mA_{m+2}]$ ($m = 1, 2, 3, 6, 9, 12$ etc.).

The first of them, $k_{1,-1} = 5.7 \cdot 10^{-14}$, is already evaluated. The next one, $k_{1,0} = 1.3 \cdot 10^{-6}$, is obtained as the origo ordinate in a $l - k_{1,-1}[A]^{-1}$ *vs.* [A] diagram (Fig. 1) and $k_{1,1} = 0.30$ is found in the same way from $(l - k_{1,-1}[A]^{-1} - k_{1,0})\,[A]^{-1}$ *vs.* [A] (Fig. 2). For evaluation of the higher constants, K_2, K_3 *etc.*, $(l - k_{1,-1}[A]^{-1} - k_{1,0} - k_{1,1}[A])\,[A]^{-2}$ *vs.* [A] is drawn in Fig. 3. The experimental points in this diagram are described by a curve $K_2 + K_5[A]^3 = 8 + 17000\ [A]^3$.

Results

The solubility of $NaAgA \cdot H_2O$ as a function of [A] (5) could be described with the constants $k_{1,-1} = 5.7 \cdot 10^{-14}$, $K_0 = 1.3 \cdot 10^{-6}$, $K_1 = 0.30$, $K_2 = 8$, and $K_5 = 17000$. Potentiometric measurements in unsaturated and in supersaturated solutions (ref. [28]) have shown that the polynuclear complexes which are formed in silver thiosulphate solutions all are of the form $Ag_mA_{m+2}^{(m+4)-}$ and that the mononuclear complex, richest in thiosulfate, is AgA_3^{5-}. The polynuclear complexes, as well as AgA_3^{5-} are thus represented in the solubility polynomial by the term $K_2[A]^2$ (the potentiometric investigation gave $K_2 = 8$) and the other constants consequently represent mononuclear complexes, thus $K_0 = k_{1,0}$, and $K_1 = k_{1,1}$. If $K_5 = 17000$, which was evaluated from the solubility measurements, was a true constant, it should approximately equal to $5k_{5,5}$ as $Ag_5A_{10}^{15-}$ is the only complex included in K_5 giving $\bar{n} = 2$, which was found experimentally (*see* Table 2 and Fig. 4). However, $Ag_5A_{10}^{15-}$ does not

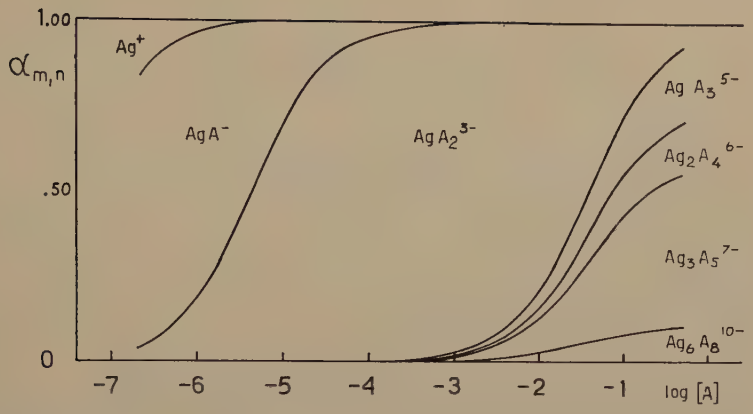

Fig. 5. The distribution of the silver on the different complexes in saturated solutions. The calculation is made according to equation (9) with the constants $k_{1,-1} = 5.7 \cdot 10^{-14}$, $k_{1,0} = 1.3 \cdot 10^{-6}$, $k_{1,1} = 0.30$, and $K_2 = k_{1,2} + 2\ k_{2,2} + 3\ k_{3,2} + 6\ k_{6,2} + \cdots = 1.91 + 1.33 + 3.90 + 0.86 + \cdots = 8.0$. If a vertical line is drawn at a certain value of log [A] the length of this line falling in the field marked Ag_mA_n gives the amount of the total silver which is present as Ag_mA_n.

satisfy the potentiometric measurements and the disagreement between the solubility determinations and the potentiometric measurements at higher thiosulphate concentrations, must be partly due to the fact that the variation of the activity factors when perchlorate ions are exchanged by thiosulphate ions operate in different ways by these two methods, and partly to the unknown diffusion potentials, for which no correction could be made. The decomposition which was observed in the solutions with $c_{Na_2A} \geqslant 75$ mM is also responsible for a part of the discrepancy, as can be seen from the measurements at $c_{Na_2A} = 267$ mM (Table 2).

In Fig. 4 log l and $\bar{n}$ are given as functions of log [A]. The concentration of the species Ag^+, AgA^-, and AgA_2^{3-} at a certain value of [A] can be directly read from the lines marked 0, I, and II. It is clear from the figure that AgA_2^{3-} dominates over the other species in a wide range of concentrations and the constant $k_{1,1}$ can accordingly be evaluated with good accuracy. The line marked S gives the sum of the silver present as $Ag_mA_{m+2}^{(m+4)-}$ ($m \geqslant 1$). The lower members in this series found by the potentiometric measurements (Ref. [28]), are AgA_3^{5-}, $Ag_2A_4^{6-}$, $Ag_3A_5^{7-}$, and $Ag_6A_8^{10-}$ with the constants $k_{1,2} = 1.91$, $3k_{2,2} = 1.33$, $3k_{3,2} = 3.90$, and $6k_{6,2} = 0.86$. To show the distribution of the silver on the different polynuclear complexes in saturated solutions, Fig. 5 has been drawn according to (9) under assumption that $K_x = 0$ for $x > 2$. The ratios between the concentrations of the different species $Ag_mA_{m+2}^{(m+4)-}$ are constant, as $k_{m,2}/K_2$ is independent of [A]. So for example the species AgA_3^{5-} always contributes $100 \cdot 1.91/8 = 24\,\%$ of the term $K_2\,[A]^2$ (marked S in Fig. 4) and it consequently always forms less than 24 % of the solubility.

The high negative charges of the big ions $Ag_mA_{m+2}^{(m+4)-}$ may be partly neutralized by sodium ions.

The influence which an ion NaA^- would have upon the evaluated constants is discussed in a following paper [28].

List of symbols

The following symbols have been used in this paper.

For the sake of simplicity S_2O_3 has been abbreviated to A.

l	solubility of $NaAgA \cdot H_2O$, total silver concentration
c_{Na_2A}	sodium thiosulphate added
c_A	$= l + c_{Na_2A}$, total concentration of thiosulfate
[A]	concentration of free thiosulphate ions
c_{NaClO_4}	sodium perchlorate added
m	number of silver atoms in Ag_mA_n
n	number of thiosulphate groups in Ag_mA_n
$\bar{n}$	average number of thiosulphate ions bound to each silver atom (8)
$k_{m,x}$	equilibrium constant (4)
K_x	sum of equilibrium constants (6)
$\alpha_{m,n}$	amount of silver present as Ag_mA_n (9)
E_m, E_0	electromotive forces (in mV) (13)
L	as defined by (15)

SUMMARY

The solubility of $NaAgS_2O_3 \cdot H_2O$ in sodium thiosulphate solutions was determined at 25°C and the concentration of free silver ions in the saturated solutions was measured by the aid of silver electrodes. Sodium perchlorate was added, to an ionic strenght of 4 *M*. From these data the following values of the constants K_x (*vide* (6) and (4)) could be evaluated.

$k_{1,-1} = (5.7 \pm 0.1)\ 10^{-14}$, $K_0 = k_{1,0} = (1.3 \pm 0.2)\ 10^{-6}$, $K_1 = k_{1,1} = 0.30 \pm 0.01$, $K_2 = 8 \pm 2$, and $K_5 = 17000$.

From potentiometric measurements in unsaturated and in supersaturated solutions (Ref. [28]), however, it was found that $K_2 = 8$ and $K_x = 0$ $(x > 2)$. The discrepancy in K_5 has been accredited to activity changes.

My thanks are due to Professor C. Brosset for his stimulating interest, to Statens Tekniska Forskningsråd which supported this work financially, and to Mr. L. E. Marthén who made the drawings.

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Molecular orbital treatment of the $3p\pi$ interaction in five-membered cyclic disulphides

By Göran Bergson

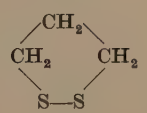
With 3 figures in the text

Introduction

To explain the fact that H_2S_2 and other divalent sulphur compounds have a skew conformation, Pauling (1), in valence bond (V.B.) language, states that the rotation barrier about the S—S bond is principally due to the $3p\pi$ sulphur electrons. The V.B. theory tells us that the repulsion between two unshared pairs of $p\pi$ electrons on adjacent atoms has a maximum when the orbitals are parallel (dihedral angle $\varphi = 0°$) and a minimum when they are orthogonal ($\varphi = 90°$). These considerations are in good qualitative agreement with the fact that the dihedral angle for a disulphide (I) (i.e. the angle between the planes R—S—S and S—S—R) is about 90° (2), since this angle is of course equal to the dihedral angle between the $3p\pi$ orbitals. In this theory the second lone pair on each sulphur atom is said to occupy mainly an s-orbital. This picture of the electronic configuration of the disulphide molecule leads to a simple twofold rotation barrier, the height of which has been estimated to about 12 ± 2 kcal/mol (3, 4).

R—S—S—R

I



II

It is easily realized that the dihedral angle in 1,2-dithiolane (II) is not far from 0° if the valence angles and bond lengths in the molecule are within reasonable limits. This abnormal dihedral angle plus some deviations in valence angles and van der Waals forces between the hydrogen atoms accounts for the fact that 1,2-dithiolane has an extra "strain energy" if compared with normal non-cyclic disulphides (5). The values found in the literature for this energy vary from 5 to about 27 kcal/mol. (6). In any case the ring strain is evidently responsible for many of the peculiarities of the dithiolane ring (5, 7). One remarkable fact is that dithiolane is yellow (absorption maximum at 3300 Å) but normal disulphides colourless (absorption maximum at 2500 Å) (5). From the chemist's point of view absorption spectra are best interpreted in molecular orbital (M.O.) language. Since the light absorption of disulphides certainly involves the $3p\pi$ electron system,

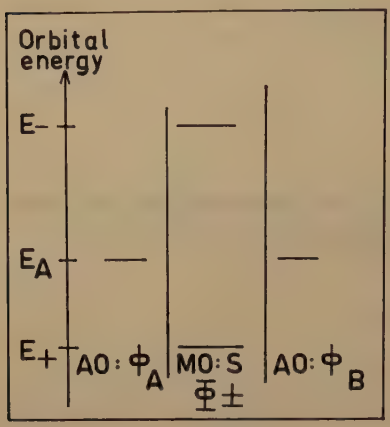


Fig. 1. Orbital energies for atomic and molecular orbitals.

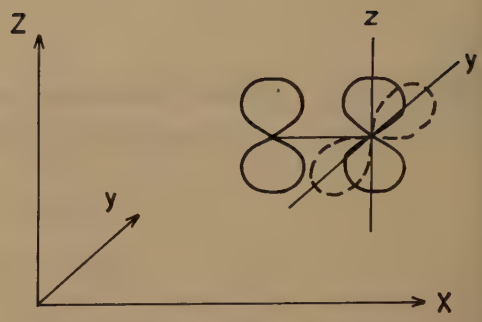


Fig. 2. Atomic orbitals p_y and p_z.

it is of interest to look for the molecular orbital counterpart of Pauling's valence bond discussion.

General theory

If we form molecular orbitals by the LCAO-method (linear combination of atomic orbitals) from the atomic orbitals ϕ_A and ϕ_B, and the atoms A and B are equal, the two possible MO:s are:

$$\Phi_{\pm} = \phi_A \pm \phi_B$$

Solution of the secular equation gives the corresponding *orbital energies* E_+ and E_-:

$$E_+ = \frac{E_A + \beta}{1+S} \qquad E_- = \frac{E_A - \beta}{1-S}$$

where

$$E_A = \int \phi_A H_{\text{eff}} \phi_A \, dv$$
$$\beta = \int \phi_A H_{\text{eff}} \phi_B \, dv$$
$$S = \int \phi_A \phi_B \, dv$$

H_{eff} is the effective Hamiltonian operator for one electron. The mathematics of this deduction is found in textbooks of quantum chemistry. The splitting of the doubly degenerate level E_A into the two states E_+ and E_- is pictured in Fig. 1. Φ_+ is termed a bonding orbital and Φ_- an antibonding. Let now ϕ_A and ϕ_B be p_y or p_z orbitals on the atoms A and B. If $\phi_A = p_y$ and $\phi_B = p_z$ (dihedral angle $\varphi = 90°$) the energy of the system is given by E_A and there is no splitting for symmetry reasons. If, however, $\phi_A = p_z$ and $\phi_B = p_z$ the energy splitting has its maximum (Fig. 2).

Now we are interested in how to detect conformation changes in the UV-spectra. We must therefore calculate the difference in the total energy, E, of the $3\,p\pi$ system in i.e. the extreme conformations corresponding to $\varphi = 0°$ and $\varphi = 90°$:

$$\Delta E_N = E_0 - E_{90}$$

N denotes the number of electrons in the system. For the ground state $N=4$ but in the excited state one electron is removed and placed in a higher orbital. As explained below, the energy of this orbital is assumed not to change with φ. ΔE_N is therefore calculated for $N=4$ and $N=3$. The total energy is the sum of the orbital energies plus a correction term which accounts for the fact that the Coulomb repulsion has been considered twice. Because this correction varies only slowly with φ it may be considered a constant which vanishes in forming the energy differences (cf. calculations on $2p\pi$ orbitals by Sponer and Löwdin (8)). The final expression for ΔE_N is therefore:

$$\Delta E_N = N_+ E_+ + N_- E_- - N E_A$$
$$N_+ + N_- = N$$

In Table 1 the expression for ΔE_N is given in column 3. For every configuration ΔE_N is proportional to $(\beta - E_A S)$ and the constant of proportionality depends only on the overlap integral S. This integral has been calculated for $3p\pi$ orbitals using Slater functions by Mulliken and coworkers (9). If the distance between the sulphur nuclei is 2.08 Å, S is 0.129. With this value of S, ΔE_N is given in column 4 where $\gamma = (\beta - E_A S)$. Since it can be shown that γ is negative (10) the sign of ΔE_N is as in column 5. We have now come to the final point of importance for the influence of conformation changes on spectra. Because that system which has the largest negative value of the energy E_N, is the most stable the sign of ΔE_N gives that value of φ (0° or 90°) which is preferred by a given electronic configuration. φ for the most stable conformation is given in column 6.

Table 1.

N	Configuration	ΔE_N	Numerical value of ΔE_N	The sign of ΔE_N	φ in the most stable conformation
3	$(\Phi_+)^2 (\Phi_-)^1$	$\frac{1-3S}{1-S^2}(\beta - E_A S)$	0.624γ	negative	0°
4	$(\Phi_+)^2 (\Phi_-)^2$	$\frac{4S}{1-S^2}(E_A S - \beta)$	-0.524γ	positive	90°

Application to disulphides

a. Qualitative interpretation of spectra

From the results in Table 1 it is evident why disulphides in their ground state tend to assume the dihedral angle 90°. We can also predict the preferred conformation for the excited state. Since the electrons in the antibonding Φ_- orbital have the highest energy of the electrons in the system it is one of these electrons which is excited in the first transition observed in the near UV. The new orbital for the excited electron is probably an antibonding σ-orbital, which can explain the fact that the S—S bond is broken when exposed to UV-light (11, 12). The energy of this orbital does not change with φ for symmetry reasons (cf. the

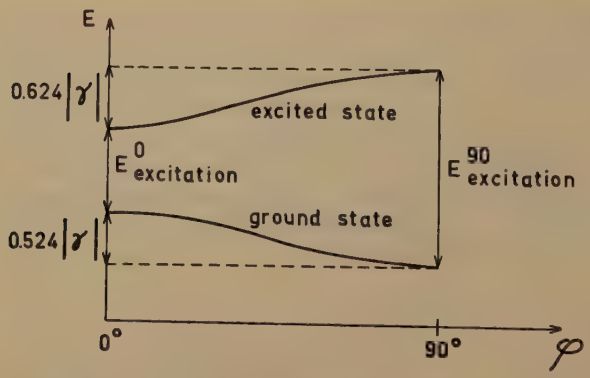


Fig. 3. The energy of the $3p\pi$ system as a function of the dihedral angle φ.

interpretation of the UV-spectra of the F_2 and Cl_2 molecules (13)). The only dihedral angle dependent part of the disulphide molecule in the first excited state is thus the $3p\pi$ system just as in the ground state. From Table 1 we can see that the first excited state has its energy minimum when $\varphi = 0°$. Fig. 3 gives approximately the energy of the $3p\pi$ electron system in the ground and excited state as a function of φ.

The excitation energy is obtained as the difference between the two curves in Fig. 3 for any given value of φ since φ does not change during the excitation (Franck-Condon's principle). In the real disulphide the energy due to deformation of valence angles and changes in van der Waals forces changes with φ but equally in the ground and excited state and as φ is constant during the excitation only the energy changes in the $3p\pi$ system are detectable in UV-spectra. The conclusion is that the red shift observed when going from a normal disulphide to 1,2-dithiolane is due to two factors: the increase in energy of the $3p\pi$ system in the ground state and the decrease in energy in the excited state, the latter being as important as the first.

b. Numerical calculations

As mentioned in the introduction the repulsion for a pair of $3p\pi$ electrons is 12 ± 2 kcal/mole. From this value and from the position of the absorption maximum (2500 Å) for a normal disulphide let us calculate the wavelength for maximum absorption for a disulphide with $\varphi = 0°$, and compare this value with the experimental (3300 Å) for 1,2-dithiolane. Refering to Fig. 3 we have the following equations:

$$E^0_{\text{excitation}} = E^{90}_{\text{excitation}} - (0.524 + 0.624)|\gamma|$$
$$0.524|\gamma| = 12 \pm 2$$
$$E_{\text{excitation}} = hc/\lambda.$$

The results of the calculation is given in Table 2.

The conclusion is that the assumption of almost pure $3p\pi$ orbitals with normal value of the rotation barrier (12 ± 2 kcal/mole) is *sufficient* for explaining the UV-spectrum of 1,2-dithiolane.

Table 2.

	$E_{excitation}$ kcal/mole	Absorption maximum (Å)
Calculated for $\varphi = 0°$	87 ± 4	3250 ± 150
Found in 1,2-dithiolane	86	3300
Found in normal disulphides	113	2500

Discussion

For many reasons the theory developed in this paper must be regarded as qualitative and not quantitative. One reason for this is that the assumption of pure $3p$ orbitals on the sulphur atoms is not correct which can be understood by a glance at the valence angles (2). But the fact that the dihedral angle is about 90° (usually somewhat larger due to steric factors) in normal disulphides tells us that Pauling's idea of a $3p\pi$ function as principally determining the dihedral angle is a good approximation. It would however be of interest to know how hybridization affects the rotation barrier. In addition it must be remembered that the dihedral angle in 1,2-dithiolane probably is not exactly 0° as assumed in this paper. The numerical data, therefore, must not be taken too seriously.

SUMMARY

The shift in absorption maximum from 2500 Å to 3300 Å when going from a normal disulphide to 1,2-dithiolane has been explained in terms of a simple molecular orbital theory.

I am indebted to Professor Arne Fredga for all the facilities placed at my disposal, and to Dr. Lennart Schotte for his kind interest in my work. I also wish to thank Dr. Per-Olov Löwdin for a valuable discussion about molecular orbital theory. A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

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3-Substituted thiophenes

Preparation and metalation of 3-methoxythiophene

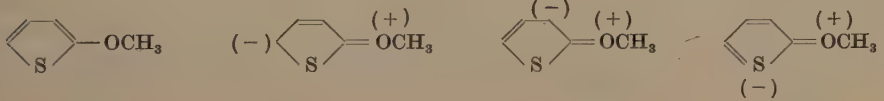
By Salo Gronowitz

With 4 figures in the text

Very few thiophene compounds containing a conjugative electron-donating substituent (+M according to Ingolds terminology) have been investigated in detail because of the great instability of such compounds as e.g. the hydroxythiophenes (1, 2) or aminothiophenes (3, 4). However, some recent investigations by Sicé (5) on 2-methoxythiophene and by Hurd *et al.* (6) on 2-acetamidothiophene show that these thiophenes, which also contain +M, −I substituents, are stable enough to be conveniently investigated. Nevertheless, hitherto with a few exceptions (4, 7) compounds with such a substituent in the 3-position of thiophene were not known.

In an earlier paper the present author and Rosenberg (8) discussed the influence of a −I, −M substituent in the 3-position of thiophene on electrophilic substitution in terms of resonance structure and pointed out that such a substituent should not deactivate position 4 towards electrophilic substitution. Molecular orbital calculations by Melander (9) has shown this to be correct.

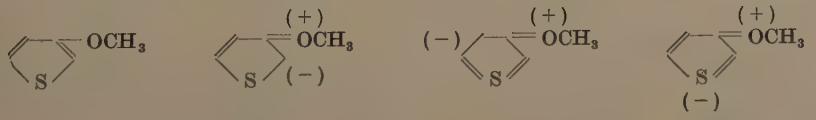
For 2-methoxythiophene the following resonance structures can be constructed.



I II III IV

Structures I, II and III may be assumed to contribute to the structure of 2-methoxythiophene. A structure as IV must have low weight, as it has a decet around sulphur as well as a C=S double bond (9). From these structures it can be concluded that electrophilic substitution should occur easiest in the 5-position, which is activated both by the sulphur and the methoxyl group, and next in the 3-position, which is activated by the methoxyl group, as has been found by Sicé (5).

For 3-methoxythiophene, only V and VI are "good" structures and it must be assumed that a methoxyl group in position 3 does not activate the 4-position in electrophilic substitution, especially as the superimposed inductive effect tends to make carbon 4 positive.



V VI VII VIII

3-Methoxythiophene is also of interest for a study of the protophilic (10) metalation with organolithium compounds. In anisole the methoxyl group is the only substituent with a free electron pair and it is assumed (11) that the metalating agent after coordination to the methoxyl group attacks one of the *ortho* protons. However, this coordination, which to a high extent facilitates metalation with organolithium compounds, modifies the effect of the substituent, and it is without meaning to speak of any directing effect, as substitution with a very few exceptions occurs in the *ortho* position. In the thiophene series, however, the sulphur atom strongly coordinates the metalating agent (12). This coordination seems to be stronger than that of the methoxyl oxygen, as Sicé (5) has found that 2-methoxythiophene is exclusively metalated in the 5-position, although nitration and acylation occurs both in position 5 and 3. In 3-methoxythiophene, if the −I effect is the most important, metalation should occur predominantly in position 2, as in the metalation of 3-bromothiophene (12). If, however, the +M-effect (which in the case of the methoxyl is not wholly a question of polarisability (13)), is the most important, then metalation should be directed toward the 5-position, as in the metalation of 3-methylthiophene, which contains a +I substituent (12).

3-Methoxythiophene was prepared in the same manner as the 2-isomer (5) by reacting 3-bromothiophene with sodium methylate in methanol in the presence of potassium iodide and cupric oxide. If the reaction is carried out with adequate stirring, a product free from 3-bromothiophene is obtained after a reaction time of 100 hours, as can be judged by the absence of the IR-peaks characteristic of 3-bromothiophene. Small amounts of 3-bromothiophene and also of 2-bromothiophene in the corresponding preparation of 2-methoxythiophene can easily be removed by treating the mixture with *n*-butyllithium at low temperature followed by hydrolysis with water. The bromothiophenes thus are converted to thiophene and pure methoxythiophenes are obtained by distillation. Distillation over sodium does not remove bromothiophene in methoxythiophene.

The substitution of bromine for methoxyl which most probably is of the nucleophilic type (14) goes without any isomerization as can be seen from the IR-spectra (Figs. 1, 2). The IR-spectrum of 3-methoxythiophene also shows the strong absorption band at 13.25 μ characteristic for 3-substituted thiophenes (15).

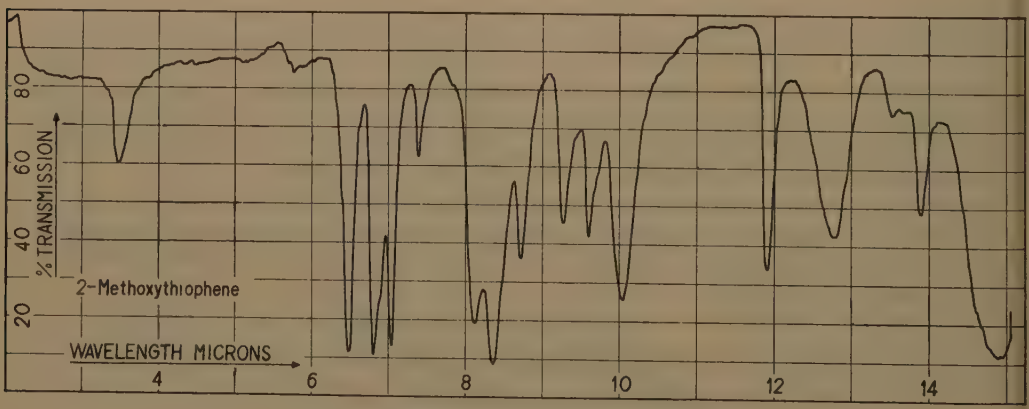


Fig. 1. IR-spectrum of 2-methoxythiophene.

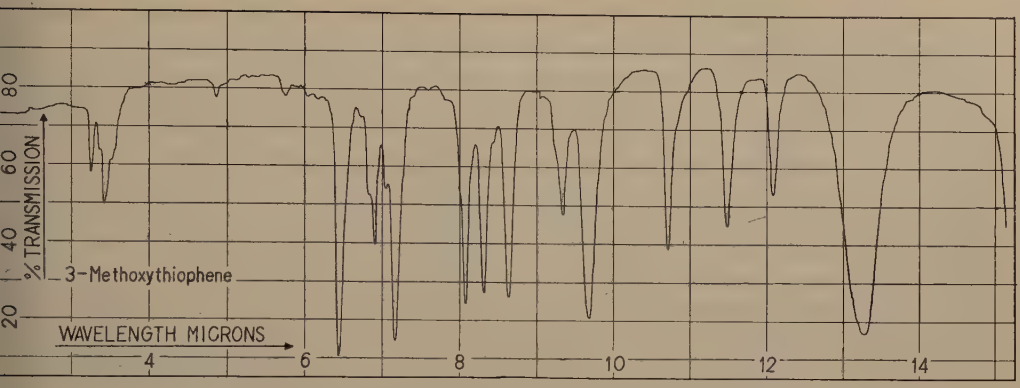


Fig. 2. IR-spectrum of 3-methoxythiophene.

3-Methoxythiophene is smoothly and rapidly metalated by *n*-butyllithium and gives upon carbonation a 3-methoxythiophenecarboxylic acid m.p. 178.5–179.5° in 86 % yield. The constitution of this acid was determined in two independent ways.

When Raney-Nickel desulphurization was used in order to determine the structure of the acid some complications occurred, as should have been expected from the work of Schwenk *et al.* (16) on the reaction of methoxyl compounds with Raney-Nickel alloy, and of Bonner (17) on the reaction with the W-2 (18) catalyst, who found that in certain cases demethoxylation occurs. The acid obtained on metalation gave on treatment with Raney-Nickel alloy an aliphatic acid which was characterized as the S-benzyl-*iso*-thiuronium salt. This salt had almost the same melting-point and gave no m.p. depression with the corresponding salt of 3-methoxyvaleric acid. In spite of this the IR-spectra of the acids and the salts showed appreciable differences. Elementary analysis indicated that the *iso*-thiuroniumsalt obtained was that of valeric acid. This result demonstrates the danger of using S-benzyl-*iso*-thiuronium salts for the identification of acids.

Thus demethoxylation occurs also during the desulphurization with Raney-Nickel alloy. Schwenk *et al.* (16) have earlier found that *ortho* and *para* methoxybenzoic acid but not the *meta* acid is demethoxylated with Raney-Nickel alloy. This could thus be taken as weak indication that the acid obtained in the metalation of 3-methoxythiophene is 3-methoxy-2-thiophenecarboxylic acid.

Sicé (5) however has found that 5-methoxy-2-thiophenecarboxylic acid upon desulphurization with Raney-Nickel catalyst W 7 (19) gives 5-methoxyvaleric acid. The desulphurization of the 3-methoxythiophenecarboxylic acid was therefore repeated with this catalyst. 3-Methoxyvaleric acid was obtained and characterized as the amide. However, some demethoxylation seems to have occurred simultaneously.

3-Methoxyvaleric acid was obtained by reacting propionic aldehyde with malonic acid in pyridine-piperidine (Knoevenagel-Doebner reaction), esterifying the obtained pentenoic acid with methanol and adding methanol over the conjugated double bond.

Another route to prove the constitution of the obtained acid was opened by recent experiments of Fiesselmann *et al.* (20) who in the reaction between esters of acetylenecarboxylic acids and of thioglycolic acid have found an elegant synthesis leading to thiophene compounds.

3-Methoxy-5-thiophenecarboxylic acid was obtained by the present author in

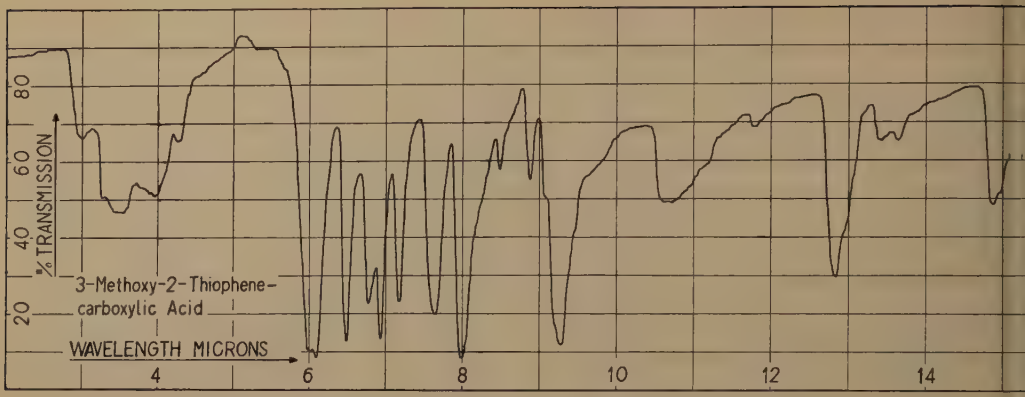


Fig. 3. IR-spectrum of 3-methoxy-2-thiophenecarboxylic acid.

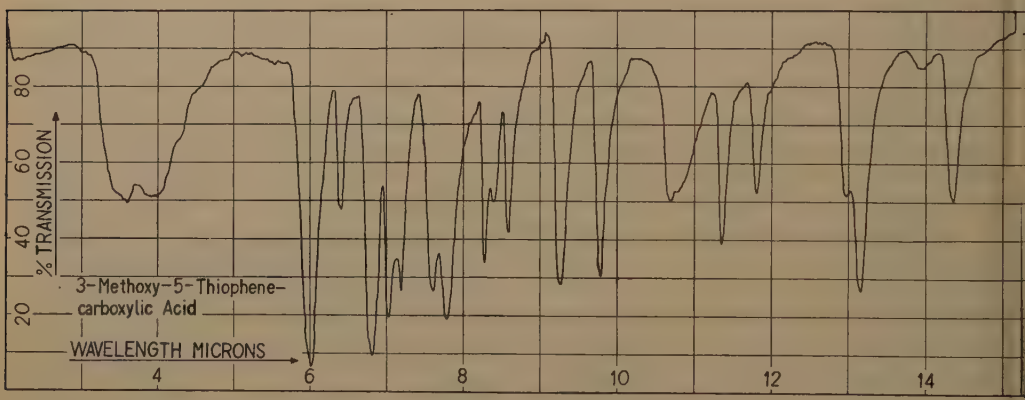


Fig. 4. IR-spectrum of 3-methoxy-5-thiophenecarboxylic acid.

the following way: The dimethylester of 3-hydroxy-2,5-thiophenedicarboxylic acid was prepared from dimethyl acetylenedicarboxylate and methyl thioglycolate in alkaline methanol (20). The ester was hydrolysed and decarboxylated to 3-hydroxy-5-thiophenecarboxylic acid, which was methylated with dimethyl sulphate, to 3-methoxy-5-thiophenecarboxylic acid. This acid was not identical with that obtained upon metalation of 3-methoxythiophene.

The route to authentic 3-methoxy-2-thiophenecarboxylic acid is more troublesome. Methyl 3-hydroxy-2-thiophenecarboxylate was prepared according to Fiesselmann *et al.* (21) through the reaction of methyl propiolate and methyl thioglycolate, a reaction which does not proceed as smoothly and with the same high yield as the corresponding reaction with the ester of the dicarboxylic acid. The obtained ester was methylated with dimethyl sulphate and saponified. The obtained acid was identical with the acid obtained in the metalation reaction. Their IR-spectra are given in Figs. 3 and 4. It has thus been shown that 3-methoxythiophene is metalated in the 2-posi-

tion. The crude acid does not contain 3-methoxy-5-thiophenecarboxylic acid as far as can be judged from the absence of its IR peaks in the product obtained.

It is thus the inductive effect which determines the place of substitution and it will be interesting to find out if the +M effect exerts any kinetic effect.

Experimental

3-Methoxythiophene.—In a three-necked flask fitted with an efficient stirrer and a reflux condenser with a calcium chloride tube, 18 g sodium was dissolved in 225 ml absolute methanol. 250 mg of potassium iodide, 45 g of 3-bromothiophene and 11.2 g of cupric oxide was added. The mixture was then refluxed with efficient stirring for 100 hours. The cooled mixture was filtered, diluted with two volumes of cold water and extracted with ether. The combined ether phases were washed with water, dried and fractionated. 25.5 g (81 %) of 3-methoxythiophene (b.p. 80–82°/65 mm Hg, $n_D^{25} = 1.5292$) was obtained.

Anal. C_5H_6OS (114.1).
calc. C 52.60 H 5.30 S 28.08;
found C 52.46 H 5.49 S 27.83.

Metalation of 3-methoxythiophene.—To 4.7 g (0.041 moles) of 3-methoxythiophene in 25 ml of absolute ether in the usual four-necked flask equipped with stirrer, reflux condenser with calcium chloride tube, dropping funnel and nitrogen inlet, 37.2 ml of 1.1-*N* *n*-butyllithium (22) solution was added dropwise and the mixture refluxed for half an hour. The solution was then poured on a mixture of Dry Ice and ether. When the temperature had risen to −10°C the mixture was hydrolyzed with water. The ether phase was extracted once with sodium bicarbonate solution. The combined aqueous phases were acidified with 2-*N* hydrochloric acid, precipitating 5.6 g (86 %) of crude 3-methoxy-2-thiophenecarboxylic acid. After one recrystallization from aqueous ethanol from which the acid crystallizes in fine white needles a m.p. of 178.5–179.5° was obtained. The acid could also be recrystallized from benzene.

Anal. $C_6H_6O_3S$ (158.2).
calc. equiv. wt. 158.2 C 45.56 H 3.83 S 20.27;
found equiv. wt. 158.7 C 45.58 H 3.85 S 20.11.

Methyl ester.—This ester was prepared in the usual manner from the acid and diazomethane in ether.

The ester was recrystallized from aqueous ethanol m.p. 54–55°. Fiesselmann (19) gives 54°.

Anal. $C_7H_8O_3S$ (172.2).
calc. C 48.82 H 4.68 S 18.62;
found C 48.83 H 4.74 S 18.56.

3-Methoxyvaleric acid.—2-Pentenoic acid was prepared according to Schjånberg (23). The presence of small amounts of the 3-pentenoic acid does not disturb in the following synthesis as only conjugated double bonds add alcohol. The acid was

esterified with methanol according to the general method given by Clinton and Laskowski (24). 48 g acid gave 45 g (86 %) ester b.p. 139–142° $n_D^{25} = 1.4402$.

To a solution of 1.5 g sodium in 70 ml of absolute methanol was added 35 g methyl 2-pentenoate. The solution was refluxed for five hours, cooled and neutralized with hydrochloric acid. The greater part of the methanol was distilled off. The residue was poured in water. The organic layer was taken up in ether. The ether phase was extracted with sodium bicarbonate solution and water and dried. Upon fractionation 24.5 g (55 %) methyl 3-methoxyvalerate. B.p. 79–82°/55 mm Hg was obtained. After another fractionation 20.5 g ester (b.p. 77–78°/55 mm Hg, $n_D^{25} = 1.4098$) was obtained which did not decolourize bromine-water. 15 g of the methyl ester was refluxed for two hours with a solution of 8 g of sodium hydroxide in 25 ml water after which time the mixture was homogeneous. After cooling the solution was extracted once with ether acidified with hydrochloric acid and extracted with ether. The ether phase, dried and fractionated, gave 8.5 g (64 %) 3-methoxyvaleric acid b.p. 120–122°/16 mm Hg, $n_D^{25} = 1.4263$.

Amide.—The crude acid chloride prepared from 2 g of the acid and 10 ml $SOCl_2$ was dropped in conc. ammonia. As no amide separated, the solution was evaporated to dryness and extracted with boiling benzene, from which the amide crystallized after cooling. After one recrystallization from benzene the amide was obtained in white flakes m.p. 85–85.5°.

Anal. $C_6H_{13}NO_2$ (131.2).
calc. C 54.93 H 9.99 N 10.68;
found C 55.10 H 9.81 N 10.67.

S-benzyl-iso-thiuronium salt.—This salt was prepared according to Vogel (25). After one recrystallization from water a m.p. of 150–152° was obtained.

Anal. $C_{14}H_{22}N_2O_3S$ (298.4).
calc. C 56.35 H 7.43 S 10.74;
found C 57.11 H 7.35 S 11.18.

Raney-Nickel alloy desulphurization.—1.0 g of 3-methoxy-2-thiophenecarboxylic acid was dissolved in a solution of 9.5 g sodium hydroxide in 50 ml of water. 13 g of Raney-Nickel alloy was added in small portions under cooling and after all the Raney-Nickel was added the mixture was carefully heated to reflux and refluxed for six hours. The hot solution was filtered and the nickel washed with a little water and the reaction product isolated in the usual manner (26). After removing the ether in vacuo a residue of 0.54 g was obtained, 50 mg of which were taken aside for IR spectrum. This spectrum showed some deviations from that of authentic 3-methoxyvaleric acid. The residue was at once converted to its *iso*-thiuronium salt. After two recrystallizations from water a m.p. of 145–148° was obtained. It gave no m.p. depression with the benzyl *iso*-thiuronium salt of 3-methoxyvaleric acid although its IR spectrum showed some differences.

It analyzes correctly as S-benzyl-*iso*-thiuronium salt of valeric acid.

Anal. $C_{13}H_{20}N_2O_2S$ (268.4).
calc. C 58.18 H 7.49 N 10.44 S 11.95;
found C 58.18 H 7.31 N 10.56 S 11.91.

Desulphurization with Raney-Nickel catalyst W 7.—The desulphurization was carried out analogously to that described by Sicé (5) for 2-methoxy-5-thiophenecarboxylic acid. 0.9 g of the 3-methoxythiophenecarboxylic acid was desulphurized, 0.4 g of acidic material (b.p. 115–120°/12 mm Hg) was obtained and converted into its acid chloride and amide in the usual manner. However, even after several recrystallizations from benzene-petrol mixtures, the amide melted 78–80°, indicating that it contained some impurity. A mixture of about equal amounts of this amide and 3-methoxyvaleramide had a m.p. of 80–82°. Its IR spectrum was identical with that of 3-methoxyvaleramide. The purification was not carried further as other proofs of the structure of the metalation product of 3-methoxythiophene could be found.

3-Methoxy-5-thiophenecarboxylic acid.—To a solution of 1.6 g sodium hydroxide in 16 ml water was added 2 g of 3-hydroxy-5-thiophenecarboxylic acid (20, 21) and 2 ml of dimethyl sulphate and the mixture vigorously shaken for half an hour at room temperature and then refluxed for two hours. After cooling, the alkaline solution was extracted once with ether. Acidification with hydrochloric acid precipitated 1.9 g of an acid, which after two recrystallizations from aqueous ethanol had a m.p. of 166–168°.

Anal. $C_6H_6O_3S$ (158.2).
calc. equiv. wt. 158.2 C 45.56 H 3.82 S 20.27;
found equiv. wt. 157.5 C 45.65 H 3.80 S 20.20.

Methyl ester.—The ester was obtained by reacting the acid in ether with an ethereal solution of diazomethane. The ester crystallized from aqueous methanol in long needles m.p. 38–39°.

Anal. $C_7H_8O_3S$ (172.2).
calc. C 48.82 H 4.68 S 18.62;
found C 49.11 H 4.74 S 18.58.

The IR spectrum of 3-methoxy-5-thiophenecarboxylic acid was different from that of the acid obtained upon metalation of 3-methoxythiophene. A mixture of equal amounts of these two acids had a m.p. of 144–150°.

3-Methoxy-2-thiophenecarboxylic acid.—0.5 g of methyl 3-hydroxy-2-thiophenecarboxylate (21) was shaken for one hour with 10 ml 2-*N* sodium hydroxide and 1.5 ml dimethyl sulphate. Then additional 1.5 ml of dimethyl sulphate and 10 ml 2-*N* sodium hydroxide were added and the mixture was shaken for an additional hour. It was allowed to stand over night and was then refluxed for three hours in order to saponify possible methyl ester. After cooling, the reaction mixture was extracted once with ether, then acidified with 2-*N* hydrochloric acid and extracted several times with ether. The combined ether phases were dried and the ether evaporated. The residue gave after recrystallization from benzene 0.27 g of an acid m.p. 175–178°, which gave no melting-point depression with the acid obtained in the metalation of 3-methoxythiophene and had the same IR-spectrum.

All melting-points were determined in a Kofler hot-stage microscope. A Perkin Elmer model 21 spectrophotometer equipped with sodium chloride prism was used for the IR absorption spectra. The solid substance were examined as solid pressed potassium bromide plates according to the method described by Schiedt and Reinwein (27).

SUMMARY

3-Methoxythiophene has been prepared. It is metalated by *n*-butyllithium in the 2-position.

ACKNOWLEDGEMENTS

The author is greatly indebted to Professor Arne Fredga for valuable discussions. Thanks are also due to the head of this institute, Professor Nils Hellström, for his kind interest and for all facilities put at the author's disposal. The IR-spectra were recorded by Mr. Stig Bergvall and the microanalysis partly by Dr. Alfred Bernhardt at Mikroanalytisches Laboratorium, Max Planck Insititut, Mülheim, and partly by Dr. A. Bengtsson of the Analytical Department of the Chemical Institute, University of Uppsala. A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

Institute of Organic Chemistry, Royal Agricultural College, Uppsala, November 1957.

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Uppsala 1958. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 12 nr 26

Communicated 8 January 1958 by Arne Westgren

Some new compounds in the system cesium oxide–boron oxide

By J. Krogh-Moe

With 2 figures in the text

Fused mixtures of cesium oxide and boron oxide in the acid range generally supercool to form a glass. Several crystalline phases not previously reported do exist, however. A study of these phases may contribute towards establishing the structure of borate glasses. Therefore a preliminary survey of phase relationships in the range from 7 to 26 mole per cent cesium oxide was undertaken.

Glasses in the system were made by fusing weighed amounts of pro analysi boric acid and pure cesium carbonate in a platinum crucible and dehydrating for a couple of hours at 1000°C. (Higher temperatures caused considerable changes in composition due to loss by volatilization.) The samples were analyzed by titrating the boron oxide with 1/10 normal sodium hydroxide solution in the presence of mannitol.

The density of the glass was determined at several compositions. Glasses used for these measurements were all quenched or rapidly cooled. The density determination was performed by weighing pieces of the glass suspended in a comparatively inert liquid (Toluene). The observed densities are plotted as a function of mole per cent cesium oxide in Fig. 1.

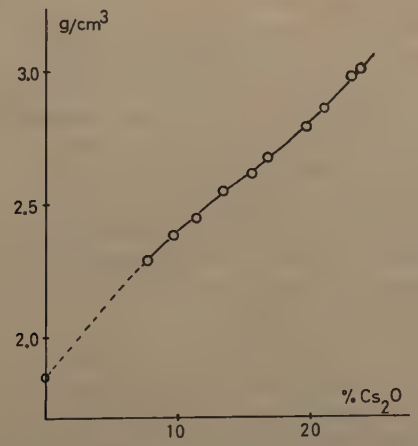

Fig. 1. Density of cesium borate glass as a function of mole per cent cesium oxide.

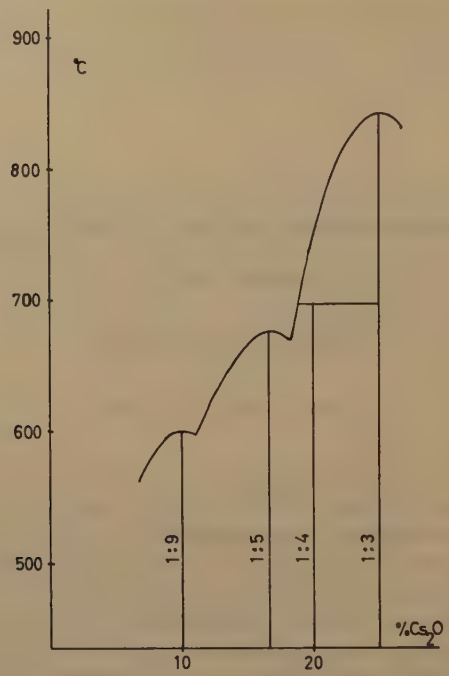

Fig. 2. Phase diagram for a part of the system Cs_2O–B_2O_3.

The furnace used for the phase equilibria experiments was controlled by a Siemens NZ temperature regulator. The temperature of the sample was measured with a Pt-Pt 10 % Rh thermocouple and a Leeds Northrup potentiometer. The glass sample, contained in a platinum crucible, was first kept at a temperature where it would devitrify. Then the temperature was raised in steps very slowly until the crystalline phase was no longer visible. No arrangement for rapid quenching was required in order to inspect the samples, since the glass exhibited an extreme reluctance to crystallize. Phase identifications were achieved with X-ray powder patterns. The accuracy of the experimental liquidus curves thus obtained are estimated to be not better than $\pm$ 15°C. A higher accuracy was not required, since the primary purpose of this investigation was to establish which crystalline compounds occur in the system. The experimental data are summarized in the phase diagram in Fig. 2.

For the crystalline phases the following crystallographic data were found. The unit cells and space groups given below were determined from single crystal weissenberg and oscillation diagrams. Densities were determined by flotation of the crystalline powder in mixtures of heavy liquids.

$Cs_2O \cdot 9B_2O_3$. Tetragonal. $a = b = 8.74$ Å, $c = 15.72$ Å. Space group either $P4_1$, $P4_3$, $P4_122$ or $P4_322$. Observed density 2.47. Calculated density 2.51 with two formula units in the cell.

$Cs_2O \cdot 5B_2O_3$. Monoclinic. $a = 5.66$ Å, $b = 9.59$ Å, $c = 6.79$ Å, $\beta = 111°$. Space group $P2_1$ or $P2_1/m$. Observed density 3.0. Calculated density 3.04 with one formula unit in the cell.

$Cs_2O \cdot 4B_2O_3$. Pseudocubic. $a = 5.74$ Å. The powder pattern can be indexed as cubic, neglecting

a few very week lines. Forbidden reflections occur. The material also exhibit birefringence. Single crystals suitable for X-ray work were not obtained.

$Cs_2O \cdot 3B_2O_3$. Orthorhombic. $a = 6.18$ Å, $b = 8.48$ Å, $c = 9.17$ Å. Space group $P2_12_12_1$. Calculated density 3.39 with two formula units in the cell.

A fifth crystalline phase appeared occasionally at temperatures around 500°C from a glass containing about 10 mole per cent cesium oxide. Somewhere between 510°C and 570°C the material recrystallized to tetragonal $Cs_2O \cdot 9B_2O_3$. The phase is monoclinic, the unit cell constants being: $a = 8.56$ Å, $b = 13.90$ Å, $c = 9.32$ Å, $\beta = 92°$. These values are not very accurate, since only single crystals giving diffuse X-ray reflections were obtained. Accurate density values proved also difficult to establish, as the crystalline phase occurred together with glass. The density is estimated to be around 2.6, however. With two formula units in the cell, this density value indicates a composition either $Cs_2O \cdot 9B_2O_3$ (theoretical density 2.72) or $Cs_2O \cdot 8B_2O_3$ (theoretical density 2.51). The similarity of lattice dimensions of the tetragonal cesium borate with this monoclinic form, indicates that the latter is probably a low temperature modification of the former and hence of less interest in connection with the study of borate glasses.

SUMMARY

Phase relationships and X-ray crystallographic data are given for five new compounds in the acid range of the system cesium oxide–boron oxide. The density of some glasses in the system was also determined.

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Stereochemical studies. III

Resolution and configuration of α-(3-indolyl)-propionic acid

By Berndt Sjöberg

With 6 figures in the text

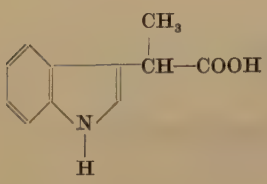
As early as 1937 Kögl (1, 2) studied the effect of optical isomerism in plant growth regulators. He found that the (+)-form of α-(3-indolyl)-propionic acid was about 30 times as active as the (−)-form in the *Avena curvature test*. However, in the *Avena cylinder test* (*straight growth test*) no essential difference was observed. According to Kögl's opinion the difference in the curvature test was due to different physiological transport of the enantiomers. It was furthermore stated that the antipodes should have the same activity if they acted directly upon the cells of the growth zone (2). Since that time a great number of optically active plant growth regulators have been investigated. In nearly all the cases stereochemical specificity has been reported (3–9).

As a clear difference in activity between the enantiomers was found even in those tests where transport effects should be eliminated, a reinvestigation of the antipodes of α-(3-indolyl)-propionic acid with the same methods as have been used in the works referred to should be of interest.

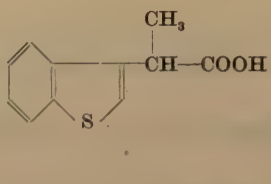
The racemic α-(3-indolyl)-propionic acid was prepared according to Kögl and Verkaaik (2). These authors also resolved the acid and obtained the (+)-form from quinine and the (−)-form from cinchonine. When using their method it was difficult to get crystalline salt from cinchonine and the antipodes were obtained in very low yield. However, in preliminary experiments it was found that the resolution could be performed in a simpler way, using brucine and cinchonidine. The dextrorotatory acid was obtained by recrystallization of the brucine salt from dilute ethanol. The cinchonidine salt gave the levorotatory acid from an ethyl acetate-methanol mixture. The specific rotation measured in absolute ethanol was $+79.4^\circ \pm 0.5^\circ$ and $-78.8^\circ \pm 0.5^\circ$; m.p. 138–140°. Kögl and Verkaaik reported $+77^\circ \pm 2^\circ$ and $-77^\circ \pm 2^\circ$ respectively; m.p. 138–139°.

A great number of optically active plant growth regulators of the general structure Ar–X–CH(R)–COOH, where X is O, S, NH or CH_2 have been investigated by Matell, and it has been shown that the D-forms have the greater activity (10). In order to find out whether there is a general connection between auxin activity and sterical configuration and if the more active forms belong to the D-series even for aralkyl carboxylic acids, attempts were made to establish the configuration of the antipodes of α-(3-indolyl)-propionic acid.

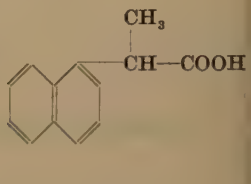
For the purpose of relating the antipodes of α-(3-indolyl)-propionic acid (I) to a substance of known configuration, comparison of the antipodes of I with the antipodes of the structurally similar α-(3-thianaphthenyl)-propionic acid (II) or α-(1-naphthyl)-propionic acid (III) by the Fredga method (11) seemed to be possible.



I



II

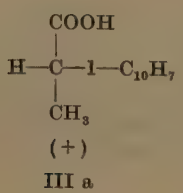


III

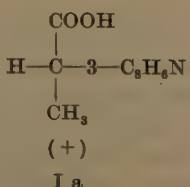
The melting point diagrams for the two component systems of the acids I, II and III were determined. The diagrams of (+)-I and (+)-II as well as of (+)-I and (−)-II are nearly identical and of common eutectic type (Figs. 1 and 2). The diagram of (+)-I and (+)-III has an eutectic point at a composition containing about 20 % of the lower melting component (Fig. 3). The acids with opposite direction of rotation give a melting point curve with an eutectic point at a composition of about 10 % of the acid III and a peritectic point at about 27 mole-% of the same acid (Fig. 4). It was possible to determine a small part of the branch representing the intermediate phase in the domain where it is instable, and the direction of this branch indicates the existence of a molecular compound in the molar ratio 1:1, which is superimposed by the higher melting component.

X-ray powder photographs confirmed that the diagrams 1–3 were of common eutectic type and that a new phase existed in diagram 4. It was furthermore possible to show that the new phase existed somewhere between 40 and 60 mole-%, because in the photographs referring to 40 mole-% lines from the naphthylpropionic acid could be discerned. At a composition of 60 mole-% of the indolylpropionic acid, lines both from the new phase and from the indolylpropionic acid could be found. The photometer curves of the X-ray powder photographs of the antipodes of α-(3-indolyl)- and α-(1-naphthyl)-propionic acid as well as of equimolar mixtures of the acids are given in Fig. 6.

It is most probable that the new phase in diagram 4 is a quasi-racemate, and in that case α-(1-naphthyl)- and α-(3-indolyl)-propionic acid with opposite direction of rotation also have opposite configuration. The dextrorotatory α-(1-naphthyl)-propionic acid has the configuration III a (8) and consequently (+)-α-(3-indolyl)-propionic acid is represented by stereoformula I a.



(+)
III a



(+)
I a

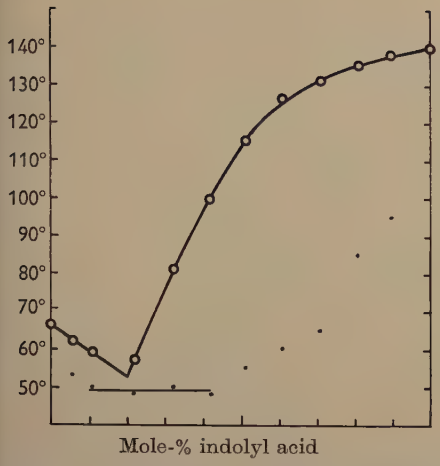


Fig. 1. (+)-α-(3-indolyl)-propionic acid and (+)-α-(3-thianaphthenyl)-propionic acid.

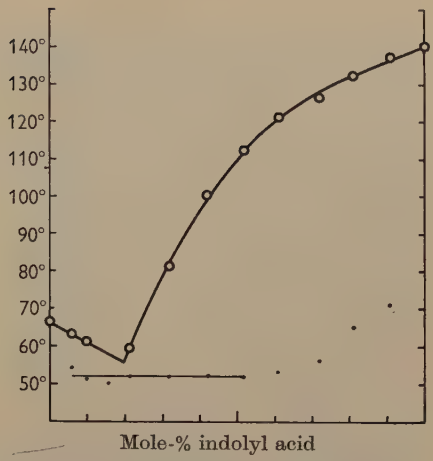


Fig. 2. (+)-α-(3-indolyl)-propionic acid and (−)-α-(3-thianaphthenyl)-propionic acid.

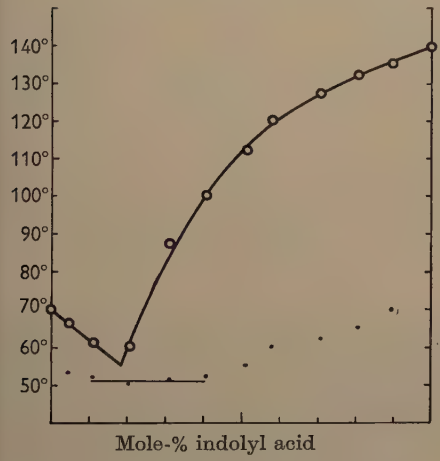


Fig. 3. (+)-α-(3-indolyl)-propionic acid and (+)-α-(1-naphthyl)-propionic acid.

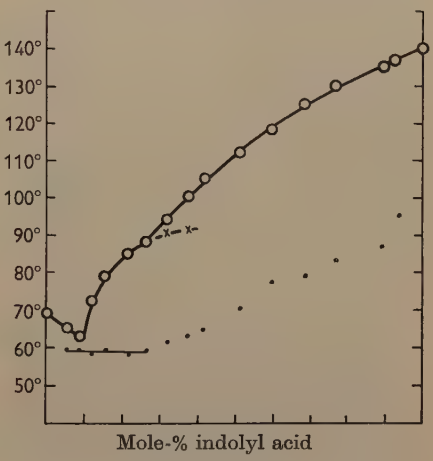


Fig. 4. (+)-α-(3-indolyl)-propionic acid and (−)-α-(1-naphthyl)-propionic acid.

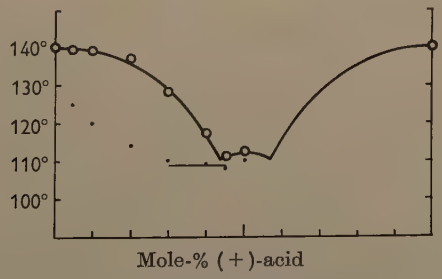


Fig. 5. (+)-α-(3-indolyl)-propionic acid and (−)-α-(3-indolyl)-propionic acid.

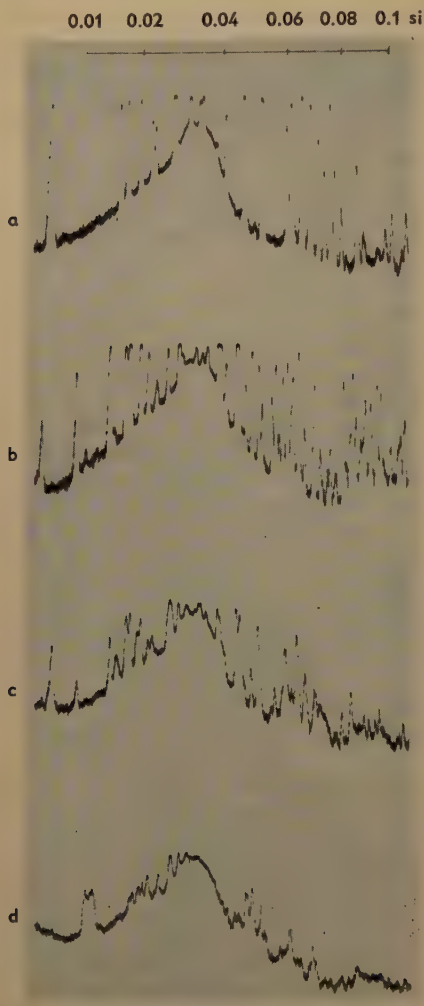


Fig. 6. Photometer curves of X-ray powder photographs of a) (+)-α-(3-indolyl)-propionic acid; b) (+)-α-(1-naphthyl)-propionic acid; c) equimolar mixture of (+)-α-(3-indolyl)-propionic acid and (+)-α-(1-naphthyl)-propionic acid; d) equimolar mixture of (+)-α-(3-indolyl)-propionic acid and (−)-α-(1-naphthyl)-propionic acid.

As α-(3-thianaphthenyl)- and α-(1-naphthyl)-propionic acid have very stable racemates (9), the reason why no quasi-racemate was formed of α-(3-thianaphthenyl)- and α-(3-indolyl)-propionic acid and why the molecular compound of α-(1-naphthyl)- and α-(3-indolyl)-propionic acid was so slightly developed may be connected with the extremely low racemate formation tendency of α-(3-indolyl)-propionic acid (Fig. 5) and the big difference in melting point of the components.

The rotatory power was determined in different solvents and in Table 1 the activity data of (+)-α-(3-indolyl)-propionic acid and (−)-α-(3-thianaphthenyl)-propionic acid are given. A very good parallelism is found, indicating that the acids with the same direction of rotation also have the same configuration (12, 13). This is in accordance with the results obtained with the quasi-racemate method.

Table 1. $[M]_D^{25}$ for (+)-α-(3-indolyl)-propionic acid (I) and (−)-α-(3-thianaphthenyl)-propionic acid II.

Solvent	$[M]_D^{25}$ I	II
Benzene	200.9°	−226.1°
2.2.4-Trimethylpentane	184°	−215°
Acetic acid	181.8°	−206.5°
Acetone	174.6°	−190.2°
Chloroform	157.8°	−171.2°
Ethanol	150.2°	−165.5°
Water (ion)	32.4°	− 2.3°

The plant growth regulating properties of the antipodes of α-(3-indolyl)-propionic acid have been reinvestigated by Professor B. Åberg who has kindly put some data at the author's disposal. The activity in the *Avena straight growth test* was considerably stronger than that of 3-indolyl acetic acid. It was confirmed that in this test both the (+)- and the (−)-antipode had the same auxin activity. However, in inhibiting the root growth of wheat the (+)-antipode was about 6 times more active than the (−)-antipode. On the other hand, in the *flax root test* the (−)-acid was about 2 times as active as the (+)-acid (14). Kögl and Verkaaik found a difference in activity between the antipodes in a test on cuttings, but they did not ascribe this test the same importance as the *Avena cylinder test* (15). In the tests on cuttings and on wheat and flax roots the transport effects should be eliminated. Undoubtedly a difference exists in the activity between the antipodes even in these tests.

Experimental

α-(3-Indolyl)-propionic acid (I)

The racemic acid was prepared according to Kögl and Verkaaik (2) in a yield ranging from 30 to 40 %.

Resolution of the racemic acid

Preliminary experiments were carried out with different bases and solvents. From the crystals formed the acid was liberated and the optical activity measured in absolute ethanol (Table 2).

A mixture of 7.6 g (0.04 mole) of racemic acid and 18.7 g (0.04 mole) of brucine was dissolved in 50 ml of hot ethanol and 20 ml of water. The salt was allowed to crystallize in a refrigerator over night and was then filtered off. Some acid was isolated from a sample and its optical activity measured in absolute ethanol. The remaining salt was recrystallized from dilute alcohol (Table 3).

The mother liquor from the first crystallization was evaporated to dryness and 3.5 g acid obtained with $[\alpha]_D^{25} = -46°$ (absolute ethanol). 3.3 g of this acid was dissolved with 5.1 g of cinchonidine in 75 ml of ethyl acetate and 5 ml of methanol. The recrystallizations were carried out according to Table 4.

Table 2. Preliminary tests on the resolution of α-(3-indolyl)-propionic acid.

Base	$[\alpha]_D^{25}$ of acid in abs. ethanol			
	From methanol	From ethanol	From acetone	From ethyl acetate
Cinchonine	oil	− 3°	− 5°	oil
Cinchonidine	− 20°	− 9°	oil	− 26°
Quinine	+ 11°	+ 6°	+ 5°	+ 4°
Quinidine	oil	oil	oil	oil
Brucine	+ 42°	+ 52°	+ 21°	± 0
Strychnine	oil	oil	oil	oil
Morphine	oil	oil	oil	oil
(+)-α-Phenylethylamine	oil	oil	oil	oil
(+)-α-(2-Naphthyl)-ethylamine	+ 8°	+ 10°	+ 3°	+ 5°

Table 3. Recrystallization of the brucine salt of α-(3-indolyl)-propionic acid.

Cryst. no.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D^{25}$ of acid
	Ethanol	Water		
1	50	20	12.3	+ 56°
2	20	5	10.0	+ 69°
3	20	5	9.3	+ 77°
4	20	5	8.7	+ 77°

Table 4. Recrystallization of the cinchonidine salt of α-(3-indolyl)-propionic acid.

Cryst. no.	Solvent (ml)		Weight of salt (g)	$[\alpha]_D^{25}$ of acid
	Ethyl acetate	Methanol		
1	75	5	7.4	− 58°
2	75	5	6.5	− 69°
3	60	4	5.6	− 75°
4	50	3	4.8	− 77°
5	50	3	4.1	− 77°

Table 5. Optical activity of (+)-α-(3-indolyl)-propionic acid.

Solvent	mg of acid	$2\ \alpha_D^{25}$	$[\alpha]_D^{25}$	$[M]_D^{25}$
Benzene	82.9	1.76°	106.2°	200.9°
2,2,4-Trimethylpentane	23.8	0.46°	97°	184°
Acetic acid	83.8	1.61°	96.1°	181.8°
Acetone	81.8	1.51°	92.3°	174.6°
Chloroform	88.7	1.48°	83.4°	157.8°
Ethanol	85.0	1.35°	79.4°	150.2°
Water (ion)	90.7	0.31°	17.1°	32.4°

(+)-α-(3-Indolyl)-propionic acid

The last salt fraction (Table 3) was treated with sulphuric acid and the organic acid extracted with ether. The crude acid (2.4 g) was recrystallized from benzene. Yield 2.2 g with m.p. 138–140°. The equivalent weight could be determined by titration with 0.1-*N* NaOH (phenolphthalein).

Anal. Equiv. wt. calc. 189.2; found 189.5.

Optical activity in different solvents

The acid was dissolved in different solvents to a volume of 10.00 ml and the rotatory power determined. The results are given in Table 5.

(−)-α-(3-Indolyl)-propionic acid

From the last fraction of the cinconidine salt there was obtained 1.4 g acid. It was recrystallized from benzene. Yield 1.2 g with m.p. 138–140°.

Anal. Epuiv. wt. calc. 189.2; found 189.6.

0.0832 g acid dissolved in absolute ethanol to 10.00 ml:
$2\alpha_D^{25} = -1.31°$, $[\alpha]_D^{25} = -78.8°$, $[M]_D^{25} = -149.1°$.

Melting point diagrams

Weighed amounts of the components were dissolved in pure acetone. The solvent was evaporated and the residue carefully powdered and dried. All melting points were determined in a hot stage microscope. The rate of heating at the melting point was about 2° per minute.

X-ray powder photographs

The photographs were taken in a focusing camera of the Guinier type with Cu-K_α radiation. The preparations were obtained in the same way as described for the melting point diagrams.

SUMMARY

α-(3-Indolyl)-propionic acid has been resolved in a new way using brucine and cinchonidine. The optical activity has been determined in different solvents and compared to that of α-(3-thianaphthenyl)-propionic acid.

The two-component systems of (+)-α-(3-indolyl)-propionic acid and the antipodes of α-(1-naphthyl)-propionic acid have been investigated. The acids with opposite direction of rotation give a quasi-racemic compound, indicating that they also have opposite configuration. As the configuration of α-(1-naphthyl)-propionic acid is known, (+)-α-(3-indolyl)-propionic acid is thus related to the D-glyceraldehyde system.

Preliminary results on the auxin effect of the antipodes are reported.

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The author is greatly indebted to Profcssor Arne Fredga for his kind interest in this work and for valuable discussions. I also want to express my gratitude to Professor Börje Åberg, Royal

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Department of Organic Chemistry, Chemical Institute, University of Uppsala, November 1957.

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Neue Amino- und Amino-Chlor-Derivate des *p*- und *o*-Xylols

Von Hans von Euler, Hans Hasselquist und Hans Wåhlstam

In Zusammenhang mit der Bearbeitung der Monoenole und der Aminophenole, die wir den Pseudo-Reduktonen zuzählen, haben wir neue Amino- und Amino-Chlor-Derivate des *p*- und *o*-Xylols dargestellt und auf ihr Verhalten gegen Tillmans-Reagens (TR), $FeCl_3$ und Nitrit sowie auf ihre Stabilität untersucht.

Amino-Derivate des *p*-Xylols

25 g 2,5-Dimethylanilin and 25 ml Essigsäureanhydrid reagieren sofort unter starker Wärmeentwicklung. Bei Abkühlung beginnt die Acetylverbindung auszukrystallisieren; nach Zusatz der 5fachen Wassermenge scheidet sich bei kräftigem Umrühren das Acetylprodukt Schmpt. 137–138° nahezu quantitativ aus.

Nitrierung.—20 g Acetamino-*p*-Xylol in 40 ml Eisessig wurden nach Zusatz von 40 ml konc. Schwefelsäure auf +10° abgekühlt und dann tropfenweise mit 20 ml konc. Salpetersäure (1,42) versetzt, so dass sich die Temp. unter 15° hielt. Nach etwa 1 Stunde begann das Nitrierungsprodukt auszukrystallisieren; nach 1 weiteren Stunde wurde mit dem doppelten Volumen Wasser verdünnt. Das vom Filtrat B getrennte Rohprodukt zeigte nach Waschen mit Wasser den Schmpt. 140°.

Aufarbeitung des Rohproduktes A.—Bei Zusatz von etwas Chloroform löst sich ein Teil der Sbst. A1 sehr leicht. Von einem schwerer löslichen Teil, der aus langen fadenförmigen Nadeln bestand, wurde abfiltriert. Die mit wenig Chloroform gewaschene Substanz (1,5 g) schmolz bei 195–196°. Bei weiterem Eindunsten des Chloroformfiltrates konnten nach 0,5 g der farblosen Nadeln isoliert werden; ihr Schmpt. war 185–191°. Durch wiederholtes Umkrystallisieren aus Eisessig stieg der Schmpt. der Nadeln auf 196,5–197°. Diese Sbst. A* konnte in 60%iger Schwefelsäure 2 Stunden auf 100° erhitzt werden, ohne dass sich ihr Charakter irgendwie änderte.

Wurde das Einengen der Chloroformlösung immer noch fortgesetzt so fielen grössere farblose Prismen aus, die nach Waschen mit wenig Chloroform in einer Ausbeute von 6 g vom Schmpt. 169–170° erhalten wurden. Bei weiterem Umkry-

* Anscheinend hat die Sbst. A 1 schon während der Nitrierung die Acetylgruppe agbespalten; in diesem Fall würde hier freies Nitramin vorliegen.

C. H. Fisher u. C. T. Walling (J.A.C.S. *57*, 1701, 1935), welche Acetamino-*p*-xylol nitrierten und hierauf durch Verseifung die Acetylgruppe direkt abspalteten isolierten 2,4-Dinitro-3,6-dimethyl-anilin mit dem Schmpt. 203–204,5° in Form *gelber Nadeln aus Eisessig*. Die beiden Substanzen scheinen nicht identisch zu sein, und eine andere entsprechende Verbindung konnten wir in der Literatur nicht finden.

stallisieren aus Benzol und dann in Chloroform wurde der Schmpt. bei 171–171,5° konstant. Sbst. A 2.

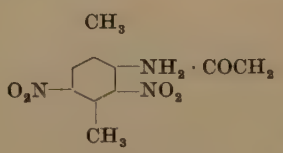
Es zeigte sich später, dass Sbst. A 2 in allen Eigenschaften mit 1-Acetamino-2,5-dimethyl-4-nitrobenzol übereinstimmte, für welches der Schmpt. 169,5–170,5 angegeben ist (2).

Aufarbeitung des Filtrates B.—Das schwach gelbe Filtrat B wurde dreimal mit Chloroform extrahiert, das sich dabei gelb färbte. Die Chloroformschicht wurde mit wasserfreiem Natriumsulfat getrocknet und im Vacuum zur Trockne verdampft. Als Rückstand hinterblieb eine schwach rotbraune Krystallmasse, die aus wenig Benzol umkrystallisiert 1 g schwach gefärbte Nadeln vom Schmpt. 185–191° ergab. Nach weiterem Umkrystallisieren aus Äthanol erwiesen sich diese Nadeln durch den Schmpt. 196–197° identisch mit Sbst. A 1.

Aus der Mutterlauge erhielten wir ausser etwas 1 A noch ein schmieriges rötliches Öl, das vermutlich 1-Acetamino-2-nitro-3,6-dimethylbenzol enthielt.

Versuch Sbst. A 1 zu identifizieren

Für die Sbst. A 1 vom Schmpt. 196–197 kommen 2 Nitrierungsprodukte in Betracht: nämlich 1-Acetamino-2,4-dinitro-3,6-dimethylbenzol, Schmpt. 228°, dargestellt von SONN (1) und 1-Amino-2,4-dinitro-3,6-dimethylbenzol, Schmpt. 202–203° (SONN) Schmpt. 203–204,5° (FISHER-WALLING, (2).



(Nach SONN)[1].

Diese beiden Substanzen lassen sich nach SONN leicht darstellen: 1 g Acetamino-*p*-Xylol wird direkt in kleinen Portionen in 6 ml kalte, rauchende Salpetersäure eingetragen. Nach etwa 5 Min. wird gerade so viel Wasser zugesetzt, dass das Nitroprodukt anfängt auszufallen. Nach 15 Min. bei Zimmertemperatur werden die gut ausgebildeten farblosen Nadeln auf einem Glasfilter abfiltriert. Die Ausbeute ist praktisch quantitativ. Nach wiederholtem Umkrystallisieren aus Äthanol wird 1-Acetamino-2,4-dinitro-3,6-dimethylbenzol in Form farbloser Nadeln vom Schmpt. 223–224° erhalten.

Der Mischschmelzpunkt dieser Substanz und Sbst. A 1 ergab 170–174°.

1-Amino-2,4-dinitro-3,6-dimethylbenzol wurden aus der entsprechenden Acetylverbindung durch 3stündige Hydrolyse in heisser wässrig-alkoholischer Schwefelsäure-Lösung erhalten. Im Verlauf der Abacetylierung fällt die freie Aminoverbindung in Form gelbroter stäbchenförmiger Nadeln aus. Nach Waschen mit Alkohol war ihr Schmpt. 206–206,5°.

Mischschmelzpunkt dieser Substanz mit Sbst. A 1 ergab 166–177°.

Da, wie erwähnt, die Sbst. A 1 sich bei der Erhitzung in schwefelsaurer Lösung als sehr stabil erwiesen hatte, wurde die Sbst. in einem neuen Versuch 7 Stunden in einem Überschuss von 20 %iger Salzsäure gekocht, wobei eine schwache Gelbfärbung der Lösung beobachtet wurde. Nach Abkühlung wurde die stark salzsaure Lösung

wiederholt mit Chloroform extrahiert, bis eine Probe des Extraktes keinen Rückstand lieferte.

Die gesammelten Chloroformschichten ergaben beim Eindunsten 60 % unveränderte Sbst. A 1 vom Schmpt. 197°.

Die salzsaure Lösung wurde mit Lauge neutralisiert und mit etwas Petroleumäther extrahiert. Nach Eindunsten auf ein kleines Volumen und Abkühlung krystallisierten beim Reiben kleine gelbrote Krystalle vom Schmpt. 36–37° aus. Dieselben erwiesen sich *identisch mit 1-Amino-2-nitro-3,6-dimethylbenzol.*

Die Sbst. A 1 dürfte somit identisch sein mit *1-Acetamino-2-nitro-3,6-dimethylbenzol.* H. WAHL (3) gibt als Schmpt. für diese Sbst. die Temp. 190° an.

1-Chlor-4-nitro-3,6-dimethylbenzol (Sbst. 268)

Darstellung.—Als Ausgangsmaterial wurde das 1-Acetamino-4-nitro-3,6-dimethylbenzol (nach anderer Bezeichnung 1-Acetamino-4-nitro-2,5-dimethylbenzol (siehe S. 260) verwendet.

5 g der Acetamino-Verbindung wurden mit 5 ml konc. Salzsäure und 10 ml Äthanol 2 Stunden unter Rückfluss auf dem Wasserbad erhitzt. Die Reaktionslösung wurde dann mit 150 ml Wasser verdünnt, wodurch anfangs das in langen farblosen Nadeln krystallisierende salzsaure Salz des freien Nitramins ausfiel, das dann durch Hydrolyse die freie Base als gelbrote Nadeln lieferte. Ausbeute 4,5 g.

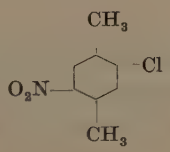
Das 1-Amino-4-nitro-3,6-dimethylbenzol, früher dargestellt von HARRISON (4), FISHER u. WALLING (2) sowie WEPSTER u. Mitarb. (5), haben wir zuerst zweimal aus Chloroform, dann zweimal aus Eissessig umkrystallisiert, und erhielten es so in reiner Form als goldglänzende centimeterlange Rechtecke vom Schmpt. 147–147,5°. Die Ausbeute an reiner Substanz, 3 g, wurde zur Darstellung der Chlor-nitro-Verbindung verwendet.

1 g 1-Amino-4-nitro-3,6-dimethylbenzol, Schmpt. 147,5° wurde nach Zusatz von 1,75 ml konc. HCl erwärmt um die Amino-Verbindung in ihr HCl-Salz überzuführen. Die Lösung wurde auf +10° abgekühlt (bei tieferer Temperatur ist die Substanz ziemlich schwer diazotierbar) und wurde dann tropfenweise mit einer gesättigten Lösung von 0,47 g $NaNO_2$ versetzt; dabei geht die Amino-Verbindung allmählich in Lösung. Nach Filtrierung wurde dieselbe unter Umrühren in eine Lösung eingetragen, die 1,2 g Cu_2Cl_2 in 5 ml konc. HCl enthielt. Die Chlorverbindung entstand sofort bei Einwirkung des Kupferchlorürs und bedeckte die Flüssigkeitsoberfläche als farbloser krystallinischer Schaum.

Die gewaschene und getrocknete Substanz 268 wurde aus wenig Petroleumäther mehrmals umkrystallisiert bis zur Konstanz des Schmpts. 80–80,5°.

Die Ausbeute betrug 1,1 g grosse farblose Prismen; dieselben sind äusserst leicht löslich in allen üblichen organischen Lösungsmitteln, schwerlöslich in Wasser.

Analyse (Sbst. 268).				
Berechnet für $C_8H_8O_2NCl$	C 51,76 %	H 4,35 %	N 7,55 %	Cl 19,10 %
Gefunden	52,30	4,70	7,37	18,83



Siehe die Umlagerung von Halogenaminen nach ORTON und JONES (6).

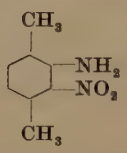
Über den *E*-effekt der CH_3-Gruppen im *p*-Xylol (Hyperkonjugation (7) und den gleichzeitigen Einfluss der Substituenten Cl und NO_2 bzw. Cl und NH_2 im Xylol ist nicht näheres bekannt.

Darstellung von 1-Chlor-2-nitro-3,6-dimethylbenzol

Da durch Nitrierung von Acetamino-*p*-xylol in Gegenwart von Eisessig keine Ausbeute an der 2-Nitroverbindung erhalten wurde, führten wir die Nitrierung nach der Vorschrift von HELDEN, VERKADE u. WEPSTER (Rec. Trav. Chim. 1.c.) unter Anwendung von nur konc. Schwefelsäure und Salpetersäure (spec. Gew. 1,40) aus. Durch Eingiessen dieser Mischung in Wasser entsteht eine Krystallmasse von Nitroisomeren, die bei 130–140° schmilzt (nach HELDEN *et al.* bei 135°).

Die genannten Autoren desacetylieren durch Erwärmen des obigen Produktes mit verdünnter Salzsäure. Nach eingetretener Hydrolyse verdünnten sie mit Wasser bis das HCl-Salz des 1-Amino-4-nitro-3,6-dimethylbenzols ausfiel und abfiltriert werden konnte.

Das Filtrat wurde auf pH 7,5 alkalinisiert und einer Wasserdampfdestillation unterworfen, wobei ein gelbrotes Öl überging, das aus 1-Amino-2-nitro-3,6-dimethylbenzol bestand. In einem Parallelversuch wurde eine kleine Menge des Öls abgekühlt und zur Krystallisation bebracht gelbrote Prismen vom Schmpt. 34–36°. Das reine Produkt schmilzt nach van HELDEN *et al.* bei 37–38°.



1-Amino-2-Nitro-3,6-Dimethylbenzol

Das ganze Wasserdestillat wurde mit Äther wiederholt extrahiert und die gesammelten Ätherschichten wurden mit konc. Salzsäure geschüttelt wobei das salzsaure Salz des 1-Amino-2-nitro-3,6-dimethylbenzols sofort ausfiel und nach Abfiltrieren und Waschen mit wenig konc. HCl bei Zimmertemperatur getrocknet wurde. Der Schmpt. des früher nicht beschriebenen HCl-Salzes beträgt 222–223°. Das Salz gibt an der Luft leicht HCl unter Gelbfärbung der Substanz ab.

In der schwach alkalischen Lösung, die durch die Wasserdampfdestillation von 1-Amino-2-nitro-2,6-dimethylbenzol befreit wurde, waren rotbraune Krystalle ausgefellen, die abfiltriert und mit wenig Wasser gewaschen wurden. Die Substanz wurde nach van HELDEN *et al.* aus Benzol umkrystallisiert, wobei eine weitere Quantität der 1-Amino-2-nitro-Verbindung isoliert werden konnte.

Aus der benzolischen Mutterlauge erhält man wenigstens ebenso viel Substanz A 1 in Form kleiner farbloser Nadeln vom Schmpt. 196,5–197°, eine Tatsache, die sich der Beobachtung von van HELDEN *et al.* entzogen hatte.

Darstellung der Subst. 271

0,6 g 1-Amino-2-nitro-3,6-dimethylbenzol HCl-Salz wurden in 2 ml Salzsäure 1:1 aufgeschlämmt und bei 0° mit einer gesättigten wässrigen Lösung von 235 mg $NaNO_2$

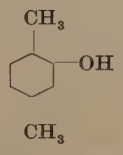
diazotiert. Diese Lösung wurde nun in 3 ml konc. HCl + 0,6 g Cu_2Cl_2 gegossen und 4 Stunden in verschlossenem Gefäss mit lose eingesetztem Glaspropfen stehen gelassen; die Stickstoffentwicklung verlief sehr langsam, während sich die Chlor-nitro-Verbindung in Form eines bräunlichen Öles abschied, das sich als *sehr flüchtig* erwies. Nach Ablauf der Reaktionszeit begann das Öl langsam in grossen Krystallblättchen zu krystallisieren. Wenn kein Öl mehr beobachtet wurde wurden die Krystalle schnell abfiltriert und mit kaltem Wasser gewaschen. Ausbeute 0,4 g.

Die Substanz konnte wegen ihrer Flüchtigkeit nicht vollständig getrocknet werden; sie wurde deswegen bei Zimmertemperatur in Petroleumäther gelöst; diese Lösung wurde mit wasserfreiem Natriumsulfat getrocknet, filtriert und im Vacuum eingeengt (durch die eintretende Abkühlung ist die Verfluchtigung der Substanz rel. gering). Die Substanz wird nun in Form grosser durchsichtiger Prismen vom Schmpt. 75–76° erhalten. Durch wiederholtes Umkrystallisieren aus etwas Petroleumäther stieg der Schmpt. der reinen Substanz auf 76–76,5°. Die Substanz 271 ist äusserst leichtlöslich in allen üblichen organischen Lösungsmitteln, schwerlöslich in Wasser. Sie sublimiert zum Unterschied von der 1-Chlor-4-nitro-Verbindung ausserordentlich leicht; schon bei Zimmertemperatur setzt sie sich an den kälteren Teilen eines geschlossenen Gefässes in Form 2 cm langer, schmaler Nadeln ab. Hat Kresol-ähnlichen Geruch.

Analyse (Sbst. 271).

Berechnet für $C_8H_{10}O$	C 78,68 %	H 8,25 %
Gefunden	78,99	8,13

Subst. 271



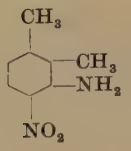
H. Wahl (Annales de Chimie (11) *5*, 27: 1936) hat in einer eingehenden Arbeit Chlor-Derivate des *p*-Xylols dargestellt und hat nach Wheeler und Morse (J.A.C.S. 46, 2572; (1925) 5-Chlor-2-Amino-1,4-Dimethylbenzol aus der entsprechenden Chlor-nitro-Produkt gewonnen.

Chlor-amino-Verbindungen des *o*-Xylols

Darstellung von 1-Acetamino-2,3-dimethylbenzol aus 2,3-Dimethylanilin und Eisessig liefert ein Rohprodukt Schmpt. 132–133° mit nahe quantitativer Ausbeute. Nach Reinigung Schmpt. 134°.

Nitrierung der 1-Acetamino-Verbindung.—Die von Wepster und Verkade (5) angegebene Methode liefert zwei isolierbare Isomere, während nach Noelting, Braun und Thesmar (8) (Nitrierung der Schwefelsäure-Lösung ohne Eisessig) die drei möglichen Mono-nitro-Isomeren erhalten werden.

Unser Nitrierungsprodukt wurde durch 2-stündiges Erhitzen mit verd. Schwefelsäure desacetyliert und dann im Wasserdampfstrom destilliert. Das dabei übergehende *1-Amino-2-nitro-5,6-dimethylbenzol* krystallisiert direkt in langen gelbroten Nadeln. Aus wenig Alkohol umkrystallisiert hat es die Form gut ausgebildeter Rhomben vom Schmpt. 120–121°.



Das rückständige *1-Amino-4-nitro-5,6-dimethylbenzol*, das nicht mit Wasserdampf übergeht wird gewonnen, wenn man die schwefelsaure Lösung ammoniakalisch macht. Die dabei entstehenden rotbraunen Krystalle werden durch Umkrystallisieren aus abs. Alkohol in Form grosser Prismen erhalten, die durch Reiben in eine gelbbraunes Pulver vom Schmpt. 116° übergingen. (WEPSTER u. VERKADE geben den Schmpt. 115,5–116,5° an, NOELTING u. Mitarb. 114°.)

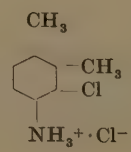
Darstellung des 1-Chlor-2-nitro-5,6-dimethylbenzols (*Sbst. 269*)

1,5 g des 1-Amino-2-nitro-5,6-dimethylbenzols wurden mit 2,5 ml konc. Salzsäure versetzt und mit 0,7 g $NaNO_2$, in wenig Wasser gelöst, diazotiert. Verdünnt man vorsichtig mit Eiswasser so verläuft die Diazotierung schnell und alles geht in Lösung. Nach Zusatz zu einer Lösung von 1,8 g Cu_2Cl_2 in 7,5 ml konc. Salzsäure entsteht die Chlor-Verbindung und fällt sofort in Form eines hellgelben krystallinischen Schaumes auf der Oberfläche aus. Dieses Produkt wird abfiltriert, mit Wasser gewaschen und wiederholt aus abs. Alkohol umkrystallisiert. Die dabei entstehenden citronengelben Nadeln schmelzen (9) bei 46–47°. Ausbeute 1,4 g. Die Subst. 269 ist leichtlöslich in allen üblichen organischen Lösungsmitteln; schwerlöslich in Wasser.

Analyse (Sbst. 269).				
Berechnet für $C_8H_8ClNO_2$	C 51,76 %	H 4,35 %	N 7,55 %	Cl 19,10 %
Gefunden	53,30	4,53	7,08	18,72

Darstellung von 1-Chlor-2-amino-5,6-dimethylbenzol (*Sbst. 270*)

0,5 g des 1-Chlor-2-nitro-5,6-dimethylbenzols wurden mit 5 ml konc. Salzsäure und 2 g $SnCl_2 \cdot 2\,H_2O$ 30 Minuten auf dem Wasserbad erhitzt, wobei das gebildete Amin als komplexes Zinnsalz in Form farbloser Prismen ausfiel. Das Abfiltrierte Komplexsalz wurde mit Salzsäure-Wasser 1:1 gewaschen und in einigen ml Alkohol aufgeschlämmt; dann liess man die mit H_2S gesättigte Lösung über Nacht unter Verschluss stehen. Nach Abfiltrieren des Zinnsulfids wurde die alkoholische Lösung auf ein kleines Volumen eingeengt und mit etwas konc. Salzsäure versetzt. (Das freie Amin erwies sich als ein Öl, das bisher nicht zur Krystallisation gebracht werden konnte.) Das Ganze wurde nun im Vacuum zur Trockne verdampft; der Rückstand, eine farblose Krystallmasse, wurde in möglichst wenig abs. Alkohol aufgelöst und vorsichtig mit Äther gefällt.



Das HCl-Salz des Amins wurde hierdurch als farblose Nadeln vom Schmpt. 231° gefällt. Ausbeute 0,2 g. Aus der Mutterlauge konnte noch mehr Sbst. 270 gewonnen werden, usw. nach Umkrystallisieren aus Alkohol-Äther mit unverändertem Schmpt.

Analyse (Sbst. 270).				
Berechnet für $C_8H_{11}Cl_2N$	C 50,02 %	H 5,77 %	Cl 36,92 %	N 7,29 %
Gefunden	49,78	5,87	36,49	7,07

Das Verhalten der untersuchten Cl, NO_2- und NH_2-Derivate des *o*- und *p*-Xylols gegen TR, $FeCl_3$ und HNO_2 sowie bezüglich ihrer Stabilität geht aus der folgenden Tabelle hervor.

Substanz	TR	$FeCl_3$	H_2SO_4-Wasser + $NaNO_2$ (10)	Cl-Abspaltung in 2-*n* NaOH 5 St. bei 80°C
p-Xylenol (Sbst. 271)	Neg.	Neg.	Erst swach braunrot, dann olivgrün	
1-Chlor-4-nitro-3,6-dimetylbenzol	Neg.	Neg.	Erst swach braunrot, dann olivgrün	Spur.
1-Chlor-4-amino-3,6-dimetylbenzol	Neg.	Neg.	Intensiv rot-violett	Spur.
1-Chlor-2-nitro-5,6-dimetylbenzol	Neg.	Neg.	Keine Färbung	Spur.
1-Chlor-2-amino-5,6-dimetylbenzol	Neg.	Neg.	Intensiv violett	Spur.

Stockholm, Universität, Institut für organisch-chemische Forschung.

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On the *trans-cis* isomerization of cinnamic acids by the action of ultraviolet rays

3-Chloro-*cis*-cinnamic acid and 3.5-dichloro-*cis*-cinnamic acid

By Sven Lindenfors

With 12 figures in the text

In 1935 Haagen Smit and Went (1) found that *cis*-cinnamic acid was an auxin and in 1951 Overbeek (2) reported that *trans*-cinnamic acid was an anti-auxin. The *cis*-acid fulfills the spatial arrangement for positive auxin activity postulated by Veldstra (3). It is well known that substitution of chlorine in an unsaturated ring may profoundly affect auxin activity. This paper is part of an investigation of chloro-substituted *cis*-cinnamic acids and deals especially with the synthetic and theoretical problems connected with photoisomerization reactions. The problem of separation of the *cis-trans* isomers has been discussed by the author in a previous paper (4).

The first photoisomerization of the unsubstituted *trans*-cinnamic acid was made by Stoermer (5) in 1909. The acid was illuminated in benzene for eight days with an Uviollamp. 25–30% was isomerized into the *cis*-form. No experimental details were given. In a paper from 1911 the same author (6) reports the photoisomerization of *o*-chloro-*trans*-cinnamic acid in water (sodium salt) and glacial acetic acid. The radiation time is long (10 days). Even in the works of Nivard (7) on *cis-trans* isomers as late as 1951 the irradiation was carried out in the same way: with long illumination times (12 days).

The first quantitative work on the photoisomerization of *trans*-cinnamic acid was done by Vaidya (8) in 1930. He showed that the quantum efficiency for the reaction *trans*→*cis* (cinnamic acid) at 313 mμ in water is 0.61 at a concentration of $3 \cdot 10^{-3}\,M$ and 0.53 at a concentration of $2 \cdot 10^{-3}\,M$. For the reaction *cis*→*trans* the quantum efficiency is 0.21 and 0.15 respectively. The UV-spectrum of Vaidya's *trans*-acid is very like that of 3.5-dichloro-*trans*-cinnamic acid as is the case for most of the chloro-substituted cinnamic acids. 3.5-dichloro-*trans* cinnamic acid has been chosen for this investigation because of its interest as a plant growth regulator. From Fig. 1 it is seen that 3.5-dichloro-*trans*-cinnamic acid absorbs very strongly ($\log \varepsilon = 4.32$) at 270 mμ. Olson (9) has shown that the line at 2537 Å in the mercury spectrum is active in bringing about the *trans*→*cis* isomerization. Monochromatic radiation cannot, however, be used when high yield is desired.

The present author has used a high pressure mercury arc, the energy distribution in the active region of which is seen in Fig. 2. Most of the energy in the ultraviolet is just in that region where the absorption from the ethylenic double bond occurs. It could thus be expected that this lamp should be highly active in bringing about

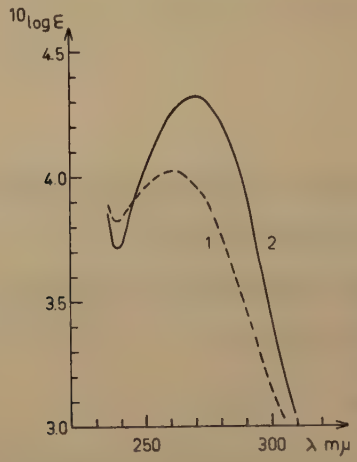
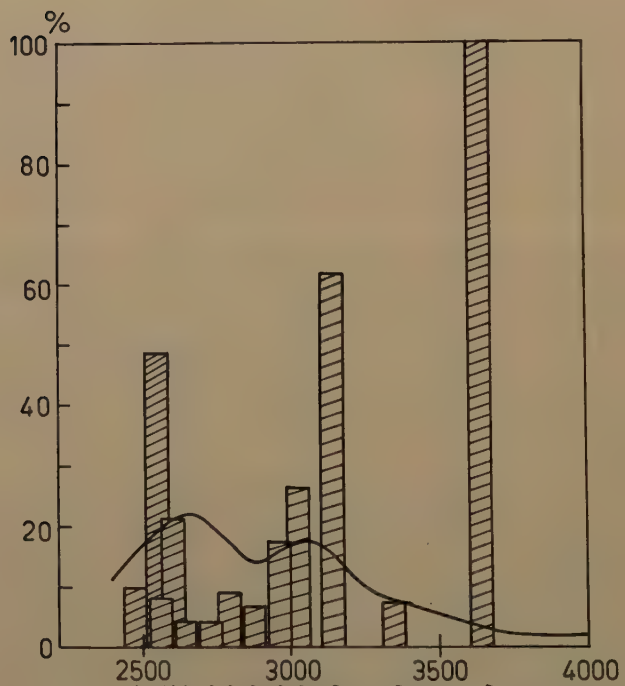


Fig. 1. Ultraviolet absorption of:
– – – – 3.5-dichloro-*cis*-cinnamic acid;
———— 3.5-dichloro-*trans*-cinnamic acid in 95 % ethanol.



Wavelength	Å	2482	2537	2653	2699	2753	2804	2894	2967	3025	3130	3341	3655
Lines	W	0.5	2.5	1.1	0.2	0.2	0.5	0.3	0.9	1.4	3.1	0.4	5.1
Wavelength	Å	2400 - 2800					2300 - 3150					3150-4000	
Continuous	W	4.4					2.5					2.3	
Total	W	8.9					8.7					7.8	

Fig. 2. Relative spectral energy distribution of the high pressure mercury quartz burner HPK 125 W (Philips').

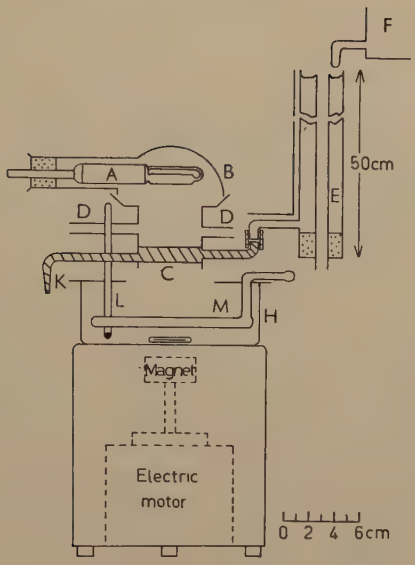


Fig. 3.

the *trans*→*cis* isomerization. The apparatus (Fig. 3) used will be described in detail in the experimental part.

From the ultraviolet spectra in solution it is of course impossible in most cases to get an idea of the fine structure from different vibrational levels which might exist on both sides of the maximum and therefore the most effective wavelength must be found empirically. For that reason short wave cut off filters have been used which show a very steep rise in transmission at a certain wavelength, Fig. 4. They are made up of lead acetate of different concentrations in water (10). The present author has found that the addition of acetic acid stabilizes the filters, *i.e.* lead is not precipitated during irradiation. Filter no. 4 (11) contains potassium acid phthalate in glycerol and water and is very stable. The curves in Fig. 4 are taken up against air in a quartz cuvette of the same thickness as that in the apparatus in Fig. 3. The eventual difference in reflexion at the surfaces of the two cuvettes are not taken into account.

By the use of these filters it is possible to get an idea of which part of the radiation is most effective for the synthesis. Fig. 5. The concentration ($4.23 \cdot 10^{-3}$ *M*) of the irradiated solutions is the same in all five cases. It is seen that when the appropriate wavelength and sufficient energy is used a very rapid isomerization can be achieved. From Fig. 5 it is seen that the time for a 50% isomerization is in the ratio of 1:4:8 for the three curves (a medium is taken for 2, 3 and 4). There is of course nothing gained by irradiation after the time at which the curves begin to go over in the very slow approach to photoequilibrium.

The mixtures at different times have been analyzed by measuring the optical density at 2700 Å. The absorption does not follow Beer's law in 95% ethanol. This might be due to dissociation irregularities. It is thus impossible to analyze the mixture from the knowledge of the molar extinction coefficient at two wavelengths. An empirical curve has been used which has been shown to give results within 1–2%. In Table 1 can be seen the optical density (D) for various mixtures of *cis*- and *trans*-

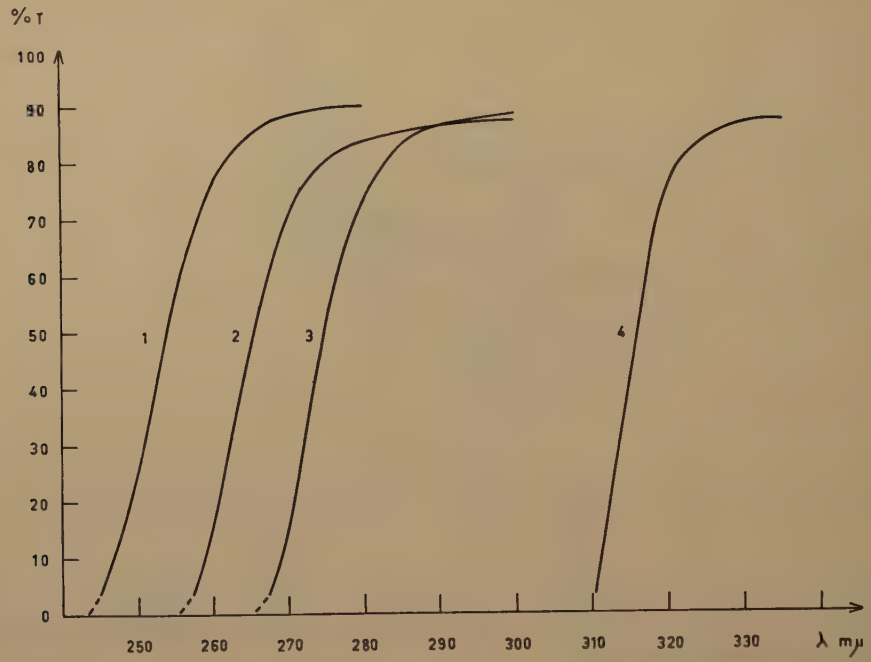


Fig. 4. Short wave cut off filters: No. 1. 1.0 g lead acette + 0.25 ml glacial acetic acid/1. No. 2. 4.8 g lead acetate + 1.5 ml glacial acetic acid/1. No. 3. 25 g lead acetate + 5 ml glacial acetic acid/1. No. 4. 40 g potassium acid phthalate in 125 ml glycerol and 1000 ml water.

3.5-dichloro-cinnamic acid at 2700 Å, in every case with a total concentration of $4.23 \cdot 10^{-5}$ *M*.

Another factor of interest from a synthetical point of view is which solvent can be used. The solvent must be stable to the active radiation and of course have rather good solubility properties. For the chloro-substituted cinnamic acids 95% ethanol is a good solvent and is also very transparent to ultraviolet radiation at the absorption wavelengths for the acids. The present author did not use ethanol in his previous study (4) on photoisomerization because of an article by Ciamician and Silber (12). These authors found in 1902 that *trans*-cinnamic acid in ethanol gave only unreacted *trans*-acid and ethylester when irradiated by sunlight for over two years. In 1914 Stoermer and Ladewig (13) showed that the esterification occurred only if traces of hydrochloric acid was present. The catalytic effect of the glass was surely sufficient to produce the ester in the investigation of Ciamician and Silber mentioned.

To see if the dielectric constant had any pronounced effect on the rate of isomeriza-

Table 1.

% cis	0	10.0	19.9	29.9	40.0	49.9	59.9	69.9	79.9	87.7	100
D_{2700}	0.830	0.780	0.733	0.700	0.671	0.616	0.579	0.535	0.485	0.450	0.395

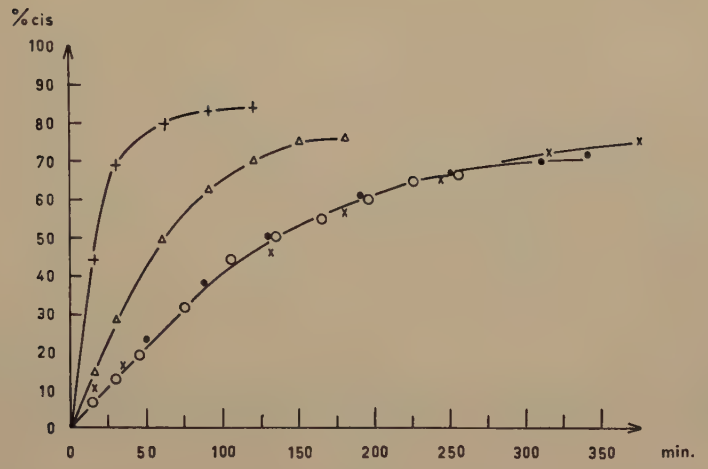


Fig. 5. Isomerization with different filters: + dest. water; △ no. 1; ○ no. 2; • no. 3; × no. 4.

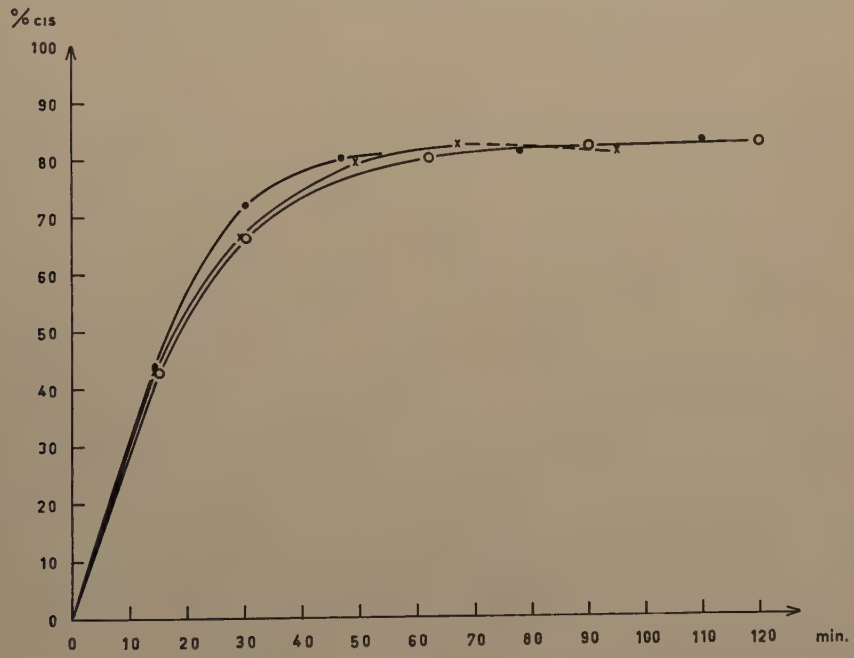


Fig. 6. Isomerization in different solvents: × benzene; ○ 95 % ethanol; • 89–91 % formic acid.

tion, irradiation was undertaken in benzene, 95% ethanol and 89–91% formic acid with the dielectric constants 2.3, 26 and 58.5 respectively. The concentration was in every case $4.23 \cdot 10^{-3}$ M. Distilled water was used as a filter. From Fig. 6 it is clear that there is no difference of practical importance under the circumstances investigated, except that the benzene solution gets a pale yellow colour after about 70

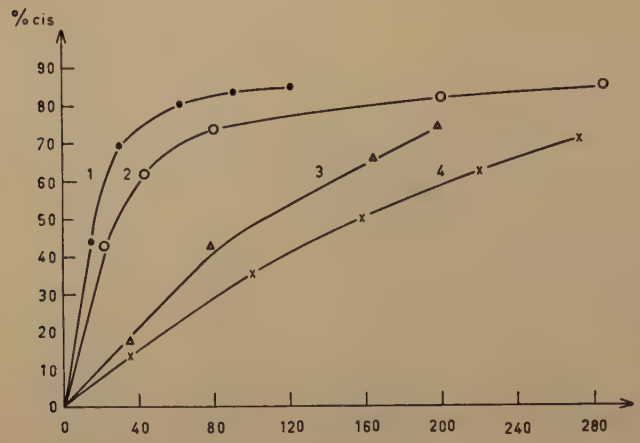


Fig. 7. Isomerization with different concentrations: 1: 1 g/l; 2: 2 g/l; 3 and 4: 5 g/l.

minutes which darkens when the irradiation is continued (broken line). The ethanol solutions are very stable and completely colourless after irradiation.

In the *trans*→*cis* isomerization two steps can be distinguished. The primary process, *i.e.* the exitation by light is a very rapid one and is often independent of temperature. The secondary reaction, in this case the step from the exited state to *cis*- or *trans*-structure, may be dependent on temperature as it is more like a thermo chemical reaction. From Table 2 it is seen that in the temperature interval +14–+40°C the temperature has a very small effect on the reaction rate in comparison with the usual "double increase in ten degrees". The photoequilibrium after two hours illumination is the same within the experimental errors.

The most important variable beside the wavelength and the intensity is the concentration. From diagrams such as those in Fig. 7 it is possible to choose the right concentration and time of illumination to get a desired amount of the *cis*-form in the shortest possible time. It is of no use to have a reaction vessel thicker than that necessary to absorb most of the radiation. As the *trans*-cinnamic acids absorb strongly, the best arrangement is to have a liquid depth of 1–2 cm and a large surface exposed to the ultraviolet rays. As the ethanol is easily driven off it is better to have relatively dilute solutions exposed for a short time than concentrated solutions illuminated for several hours. Both curve 3 and 4 in Fig. 7 correspond to 5 g/l but but for curve no. 3 a new lamp with exactly the same dimensions as the old one but with an increased energy output has been used. The greatest increase is for the lines 2537 and 2653 Å where it amounts to 44 and 45% respectively. The increase in % conversion/min is about 30 between 100 and 180 minutes. The total increase of energy in the lines between 2482 and 3130 Å is about 30%.

Table 2.

Temp. °C	14	28	40
Time for 60 % conversion in min.	30	26	32

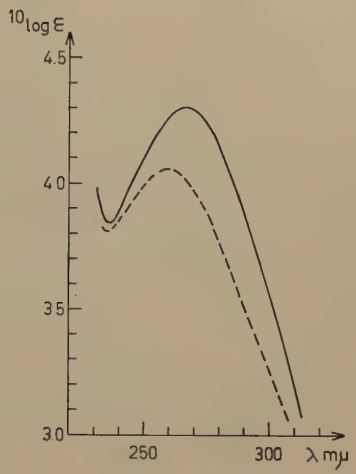


Fig. 8. Ultraviolet absorption of: – – – 3-chloro-*cis*-cinnamic acid; ——— 3-chloro-*trans*-cinnamic acid in 95 % ethanol.

Finally, the influence of stirring in the reaction vessel has been studied. In all experiments mentioned hitherto a constant stirring has been used. When a static system is used the rate of isomerization in an 1.3 cm thick solution is decreased about 10%.

To check if any byproducts have been generated during the irradiations one part of the final solution has been titrated with sodium hydroxide and another part with bromine in carbontetrachloride. In that way both the acid functions and the amount of double bonds have been checked. The results in all cases are within ± 1.5 and $\pm 3.0\%$ respectively.

From this investigation it is clear that many wavelengths inside the absorption band from the ethylenic double bond are highly active in bringing about the *trans*→*cis* isomerization. The absorption maximum corresponds to the most probable excitation which often is from the lowest vibrational level in the ground state to the corresponding vibrational level in the excited state. During the short time for electron-excitation (about 10^{-15} sec) the atoms have scarcely moved (Franck-Condon principle). But when the π-electrons are excited there is a single carbon-carbon bond left momentarily and the two carbon atoms can vibrate with a greater amplitude. This means that the excitation is to a higher vibrational level with a higher ΔE. This may be the case on the short wave side of the absorption maximum. The π-electron excitation around the absorption maximum is a singlet, that is the antiparallel spins are retained, and is accompanied by a high activation energy and a high frequency factor (14). There is also another path for the isomerization which is less probable. On the long wave side where the absorption is small the excited state may go over a triplet state (i.e. with parallel spins). As this process is rather improbable, however, it is accompanied by a low frequency factor. However, as shown by Reid (15) it is possible for a singlet state with high vibrational energy to change over to a low energy triplet. Such a reaction may have high quantum efficiency even on the long wave side. Theoretical papers on such isomerization reactions are found in (9), (16), (17) and (18).

From Figs. 1 and 8 it is seen how little the UV-spectra in 95% ethanol differ for the *trans*-isomer of 3.5-dichloro- and 3-dichloro-cinnamic acid. The *cis*-forms have lower absorption and λ_{max} is displaced about 80 Å to shorter wavelengths. 3-Chloro-

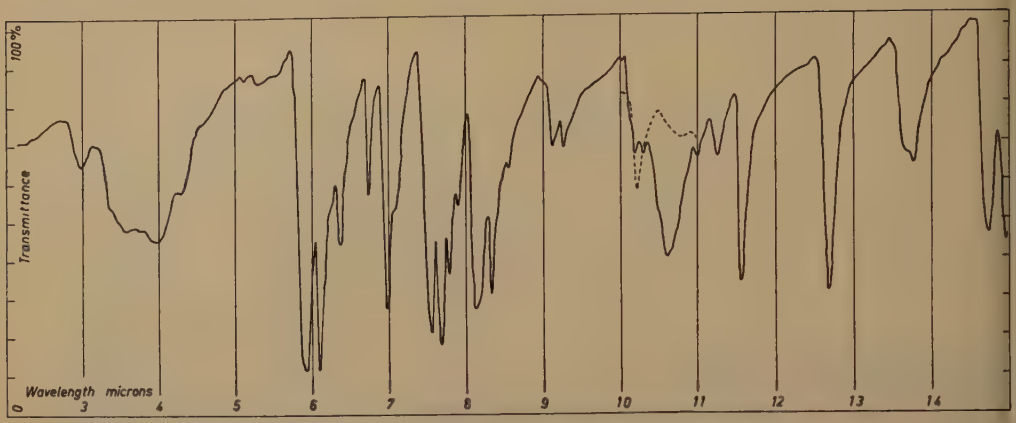


Fig. 9. IR absorption curve of 3-chloro-*trans*-cinnamic acid in KBr phase (———) and in CS_2 (– – –)

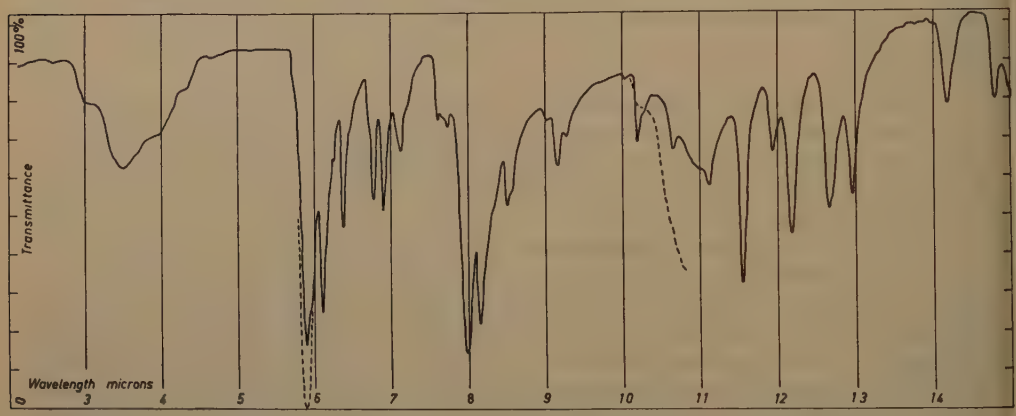


Fig. 10. IR absorption curve of 3-chloro-*cis*-cinnamic acid in KBr phase (———) and in CS_2 (– – –).

cis-cinnamic acid has been synthesized under circumstances which have been found suitable for the preaparation of 3.5-dichloro-*cis*-cinnamic acid. The *cis*-isomers have been separated from the *trans*-forms by ion-exchange chromatography with some simplifications from the method outlined by the author in a previous paper (4). These simplifications are given in the experimental part.

In Figs. 9–12 are given the infrared spectra of *cis*- and *trans*-3-chloro-cinnamic acid and *cis*- and *trans*-3.5 dichloro-cinnamic acid. The bond at about 10.2 μ which is characteristic for trans-structures (4) is very weak in the case of *trans*-3-chloro-cinnamic acid. As the acid was examined in solid pressed potassium plates this failure to appear is surely due to crystal effects. When the acid was examined in CS_2 the bond appeared with the usual strength at 10.22 μ. Surprisingly a peak appeared at 10.19 μ in the spectra for the *cis*-form but disappeared when examined in CS_2. *Trans*-3.5-dichloro-cinnamic acid in the solid phase showed the usual peak at

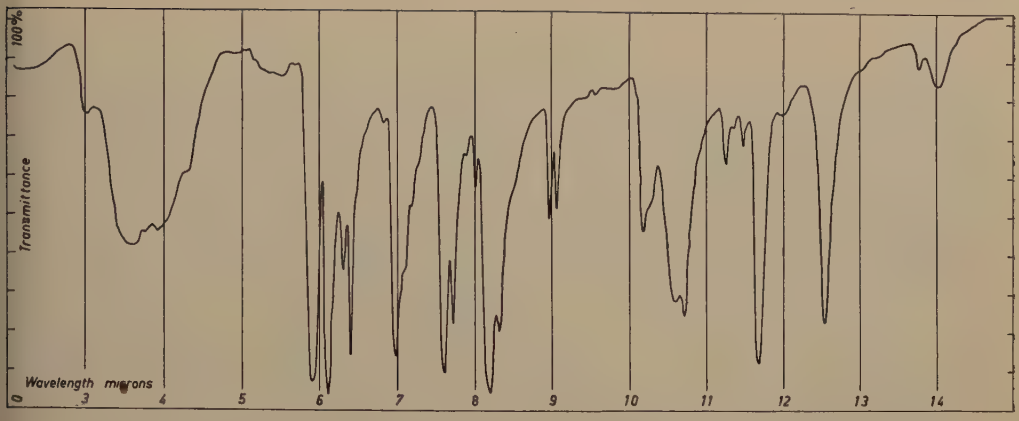


Fig. 11. IR absorption curve of 3.5-dichloro-*trans*-cinnamic acid in KBr phase.

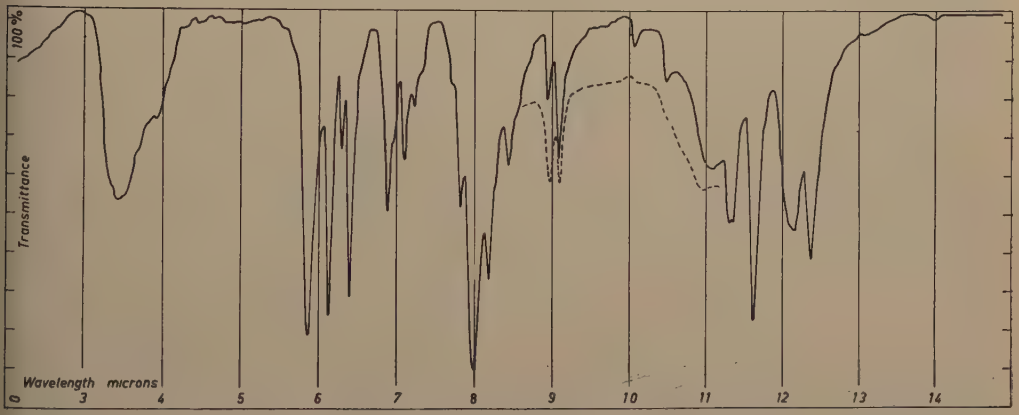


Fig. 12. IR absorption curve of 3.5-dichloro-*cis*-cinnamic acid in KBr phase (———) and in CS_2 (– – –).

10.17 μ. The small peak for the *cis*-form at 10.05 μ is eliminated when examined in CS_2. Hence it is clear that the substance must be investigated in a solvent which has no absorption around 10 μ in order that the structure may be verified.

From the biological examination made by Professor B. Åberg (19) of the geometrical isomers of cinnamic acid, 4-chloro-, 2.4-dichloro and 3.5-dichloro-cinnamic acid it is clear that in all cases the *trans*-form is a typical anti-auxin. The *cis*-forms of unsubstituted, 4-chloro-, and 2.4-dichloro-cinnamic acid are typical auxins of moderate strength but in the case of 3.5-dichloro-*cis*-cinnamic acid, which also shows positive auxin activity, some interesting complications are found at low concentrations. The properties of the isomers of 3-chloro-cinnamic acid and 2.6-dichloro-cinnamic acid (the latter of which are under preparation) will also be investigated for their auxin activities. The results will be published in a separate paper by Professor B. Åberg.

Experimental

Apparatus and general procedure.—The source of radiation was a quartz mecury burner, Philips HPK 125 W (A in Fig. 3) operated at 1.15 amp. and 125 volts. The new source mentioned before was a Philips HPK 125 WR, operated at 1.7 amp. and 95 volts. The lamp was enclosed in an aluminium reflector (B) made of a round bottom flask half of which is ground away and the interior aluminized *in vacuo*. Under the lamp hangs a cylinder (5 cm in diameter) of thin brass which contains a filter (C) of quartz (best fitted to the brass by PbO in glycerol) and one inlet and one outlet tube (D) for circulation of water which takes up the thermal radiation. As the filters used are not stable under short ultraviolet radiation for a longer time they must be renewed about every 15 minutes. That is done as shown in the Fig. (F) is a Mariotte's bottle which is convenient when the illumination takes a long time. In front of (E) is a shelter against stray-radiation. The solution to be radiated is kept in a dish (H) which has a large surface. The dish is fitted with a plate (K) holding a thermometer (L) and a glass-spiral (M) which is only used when it is desirable to have another reaction temperature than room temperature. The opening for the radiation in (K) has a diameter of 7.5 cm.

In the experiments 200 ml of $4.23 \cdot 10^{-3}$ *M* solution was radiated. To analyze the mixture after a given time of illumination 1.00 ml was taken out with a pipette calibrated to deliver 1.000 ± 0.002 ml ethanol. 1.0 ml of a solution containing 150 mg sodiumchloride in 25 ml of water and 225 ml of 95% ethanol was added and diluted to 100.00 ± 0.03 ml with 95% ethanol. The sodiumchloride has proved to give a smoother calibration curve than ethanol alone. The solution was measured in comparison with ethanol (without NaCl) at 2700 Å in calibrated cells. In the cases where benzene and formic acid were used the sample taken was diluted to 100 ml with 95% ethanol (and NaCl-solution) and measured against a solution containing 1.00 ml benzene or 1.00 ml formic acid in 99 ml 95% ethanol. The results from measurements on known mixtures of *cis*- and *trans*-acids in benzene and formic acid agree within $\pm 2\%$. For every ml removed from the reaction vessel the same amount of the stock solution was added. This introduces no error as the measurements are made with rather long time intervals. For this method to give accurate results the volume must be kept constant and that was done by hanging the dish on one arm of a laboratory balance (not shown in the Fig.). When weighing, a 2 mm plate of soft iron was introduced between the dish and the magnet to screen the magnetic field. In that way the volume was kept constant to ± 0.2 ml at the measurements.

Titration of the double bond

After the irradiation a volume containing about 55 mg acid was taken out the ethanol was driven off and the residue dissolved in 10 ml CCl_4 (p.a.). The solution was transferred to a Pyrex glass bottle fitted with a glass stopper and gently warmed until the stopper had jumped some time to expell the air. 5.00 ml 0.1295 *N* Br_2 (p.a.) in CCl_4 was added and the bottle was irradiated with a HPK 125 W lamp without reflector at a distance of about 2 dm for 10 minutes. 10 ml of water and about 2 g KJ was added. The bottle was shaken until the potassium iodide was dissolved. The generated iodide was titrated with 0.1024 *N* sodium thiosulfate. CCl_4 acts as a very sensitive indicator.

3-Chloro-trans-*cinnamic acid*

The acid was synthesized according to Doebner's method. 14 g (0.1 mole) 3-chlorobenzaldehyde (20), 15.6 g malonic acid (0.15 mole), 4 ml pyridine and 0.2 ml piperidine are warmed on the water bath for 2 hours and then in an oil bath to about 160°C for about 15 minutes. The product is poured into ice cold conc. hydrochloric acid and then filtered off. The yield of crude product is 16.5 g (91%). The product is recrystallized from 85% formic acid and 95% ethanol, m.p. 163.5–164.5°. Matell (21) gives the m.p. 163–164.5°. All melting points in this paper have been determined according to the microscopical methods of Kofler and Kofler (22). The apparatus used was a Reichert hot stage microscope.

Anal Subst., 173.6 mg: 9.46 ml of 0.1001 *N* NaOH
Equiv. wt. Calc. 182.60. Found 183.3.

3-Chloro-cis-*cinnamic acid*

A solution of 2 g *trans*-acid in one liter 95% ethanol was irradiated in 200 ml portions for 60 minutes each, without any filter but with water flowing through D. The ethanol was driven off and the product extracted with 4 × 45 ml boiling petroleum ether. 1.3 g of about 90% *cis*-acid was obtained. The *cis*-form was purified as has been described for 4-chloro- and 2.4-dichloro-*cis*-cinnamic acid (4) with the following changes: the column was loaded with 0.4 g in 45 ml 75% ehtanol instead of 0.2 g, the *trans*-acid was eluted with 0.005 *M* HCl instead of 0.004 *M* and *cis*-form was eluted with a 0.3 *M* sodium chloride solution in 75% ethanol. The elution was followed by diluting the fractions with 0.3 *M* sodium chloride solution in 75% ethanol and measuring D at 2670 and 2800 Å. When D_{2670}/D_{2800} reaches about 2 pure cis-form comes out. Recrystallized once from petroleum ether, m.p. 65.0–66.5°.

Anal. Subst., 104.3 mg: 5,69 ml of 0.1001 *N* NaOH
Equiv. wt. Calc. 182.60. Found 183.1.

3.5-Dichloro-trans-*cinnamic acid*

This acid was synthesized in the same way as 3-chloro-*trans*-cinnamic acid from 17.5 g (0.1 mole) 3.5-dichloro-benzaldehyde. The aldehyde was synthesized according to Asinger and Lock (23). It is a six steps synthesis which is very tedious. The present author has worked out a much simpler way of synthesizing this product. This results will be published in a later paper. The yield of crude acid was 20.6 g (95%). The product was redrystallized from 85% formic acid and 95% ethanol, m.p. 174.5–175.5°. Asinger and Lock give 176°.

Anal. Subst., 204.6 mg: 9.40 ml of 0.1001 *N* NaOH.
Equiv. wt. Calc. 217.05. Found 217.4.

3.5-Dichloro-cis-*cinnamic acid*

A solution of 2 g *trans*-acid in one liter 95% ethanol was irradiated in 200 ml portions for 60 minutes each, without any filter but with water flowing through D. The ethanol was driven off and the product extracted with 4 × 50 ml boiling petroleum ether. 1.25 g of about 95% *cis*-acid was obtained. The *cis*-form was purified

in the same way as described for 3-chloro-*cis*-cinnamic acid except that 0.004 *M* HCl was used instead of 0.005 *M*. The elution was followed by measuring D at 2600 and 2800 Å. Recrystallized once from petroleum ether, m.p. 113.2–115.0°.

Anal. Subst., 106.2 mg: 4.91 ml of 0.1001 *N* NaOH.
Equiv. wt. Calc. 217.05. Found 216.3.

All measurements in the ultraviolet have been made with a Beckman Model DU Spectrophotometer.

A Perkin Elmer model 21 spectrophotometer equipped with sodium chloride prisms was used for the infrared absorption measurements. The acids were examined as solid pressed potassium bromide plates and in some cases in CS_2. The calibration of the absorption curves is correct to $\pm 0.01\ \mu$ at $10\ \mu$.

SUMMARY

The method of synthesizing *cis*-forms of cinnamic acid type by the action of ultraviolet rays has been systematically studied. Wavelength, intensity, concentration, solvent and temperature dependence has been studied.

It has been shown that photoisomerization is very effective when properly used for the synthesis of *cis*-forms wich hitherto have been prepared by great difficulty.

An apparatus which has shown to be very useful for synthetic work has been constructed.

3-Chloro-*cis*-cinnamic acid and 3.5-dichloro-*cis*-cinnamic acid wich are new substances have been prepared in good yields.

The UV- and IR-spectra for the four isomers are given.

Preliminary experiments on the auxin effect of some *cis-trans*-isomers are reported.

ACKNOWLEDGEMENTS

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This investigation has been supported by *Jordbrukets Forskningsråd* to which acknowledgement is gratefully made.

Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, October 1957.

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Tryckt den 28 mars 1958

Uppsala 1958. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 12 nr 30

Vorgelegt am 13. Oktober 1954

Verlauf der Bildung und Umwandlung von Reduktonen in alkalischer Lösung

Von Maria Luisa Stein und Hans von Euler

Mit 5 Figuren im Text

Für die Darstellung des Triose-Reduktons (Reduktons I) aus Glucose in Gegenwart von Bleiacetat (Alkalinität 0,3 *n*. NaOH) hat sich die Temperatur von 90° als geeignet erwiesen. Aus einer 3 Minuten lang so behandelten Lösung wurde mit Essigsäure die Bleifällung vorgenommen.

Das aus Glucose so gebildete Redukton I ist aber in alkalischer Lösung von 0,3 *n*. NaOH über etwa 65° *nicht stabil*. Bei 75° nimmt die Endiol-Reaktion der Lösung schnell ab, wie die Fig. 1 zeigt, in welcher die nach zunehmenden Reaktionszeiten noch vorhandenen Äquivalente Endiol per 100 Mol Glucose aufgetragen sind.

Schon nach 10 Minuten ist das Maximum an Endiol erreicht, dann fällt die Kurve steil ab. Hierbei muss berücksichtigt werden

1) dass die alkalische Glucoselösung ausser dem Redukton I noch *wenigstens ein* weiteres Redukton enthält, und dass

2) die für jede Zeit gemessenen und in Fig. 1 eingetragenen Werte die Ergebnisse sind aus den in dieser Zeit gebildeten und in der gleichen Zeit wieder verschwundenen Endiole.

In welchem Betrag Redukton I unter den gleichen Versuchsbedingungen verschwindet lässt sich aus der Fig. 2 entnehmen.

Was die Art der Veränderungen betrifft, durch welche die Endiolgruppen bei Erhitzen in der 0,3 *n*. Natronlauge verschwinden, so lassen sich dieselben nur zum kleinen Teil auf Polymerisationen zurückführen.

In Fig. 1 sind nicht nur die unmittelbaren Messungsresultate der TR-Titration eingezeichnet, welche den wirklichen Endiolgehalt der Lösungen anzeigen, sondern auch diejenigen Werte eingetragen und in einer Kurve (— — — —) vereinigt, welche sich berechnen lassen, wenn man die gefundenen Endiolgehalte auf Grund der in Fig. 2 angegebenen Werte korrigiert. Diese berechnete Kurve zeigt, dass nach etwa 10 Minuten ein etwa 30 % höheres Maximum der Endiolbildung eintreten würde, wenn nicht gleichzeitig ein Teil der gebildeten Endiolgruppen auch wieder zerstört würde.

In diesem Zusammenhang mag übrigens Folgendes bemerkt werden: Die schon 1933 angegebene Tatsache, dass im ganzen auf 1 Mol Glucose 1 Äquiv. Endiol durch TR-Titration nachweisbar ist, kann so gedeutet werden, dass jedes Glucose-Molekül in der alkalischen Lösung *wenigstens intermediär* 1 Äquivalent Endiol bildet, und es steht also die Möglichkeit offen, dass man durch ein geeignetes Abfangeverfahren das Redukton I in annähernd 100 %iger Ausbeute erhalten kann, sofern die gesamte

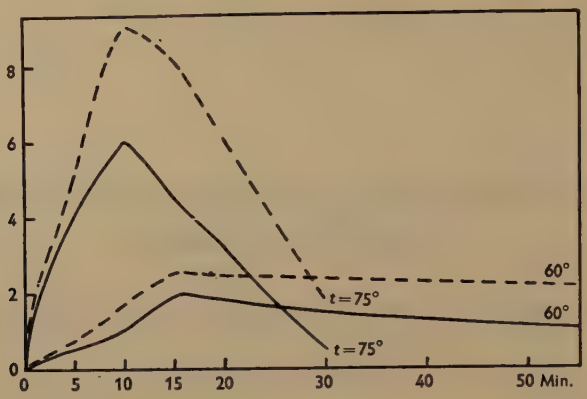


Fig. 1. Äquivalente Endiol/100 Mol Glucose.
Äquivalente Endiol aus Glucose beob. ———,
Äquivalente Endiol aus Glucose korr. – – – –.

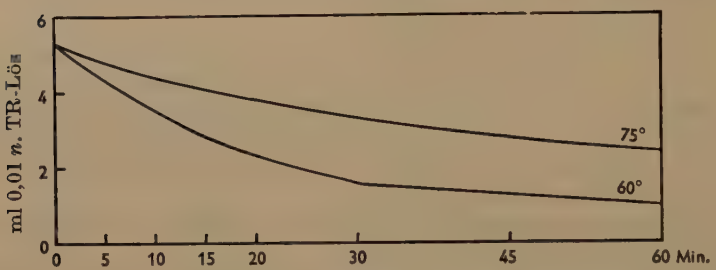


Fig. 2.

gemessene Endiolbildung vom Redukton I herstammt. Dies ist aber, wie sich in letzter Zeit gezeigt hat[1], nicht der Fall.

Welche Stoffe sich bei der Behandlung der Glucose mit mässigen Alkalikonzentrationen (etwa 0,3 *n*.) ausser den Reduktonen bilden, bleibt noch zu untersuchen.

Alkalische Behandlung der D-Xylose

Bei Untersuchungen aus diesem Institut hat sich bereits wiederholt ergeben, dass bei der TR-Titration von D-Xylose sich nicht, wie bei der Glucose, 1 Endiol auf 1 Mol Zucker bildet, sondern 2 Endiole. Bei der (nicht-oxydativen) alkalischen Behandlung liefert, unter vergleichbaren Versuchsbedingungen, die D-Xylose etwa 40 % mehr Endiol als D-Glucose.

Nach Versuchen von Hasselquist[2] wird bei der TR-Titration von Xylose in warmer (65°) alkalischer Lösung so viel TR verbraucht, „wie wenn sich aus *einem* Mol der Pentose *zwei* Endiol-Gruppen bilden würden".

[1] EULER u. H. HASSELQUIST, Virtanen-Festschrift, Suomalainen Tiedeakademia, im Druck.
[2] EULER und H. HASSELQUIST, Experientia *5*, 51; 1949 (Karrer-Festschrift). Siehe auch Reduktone, Stuttgart 1950, S. 14.

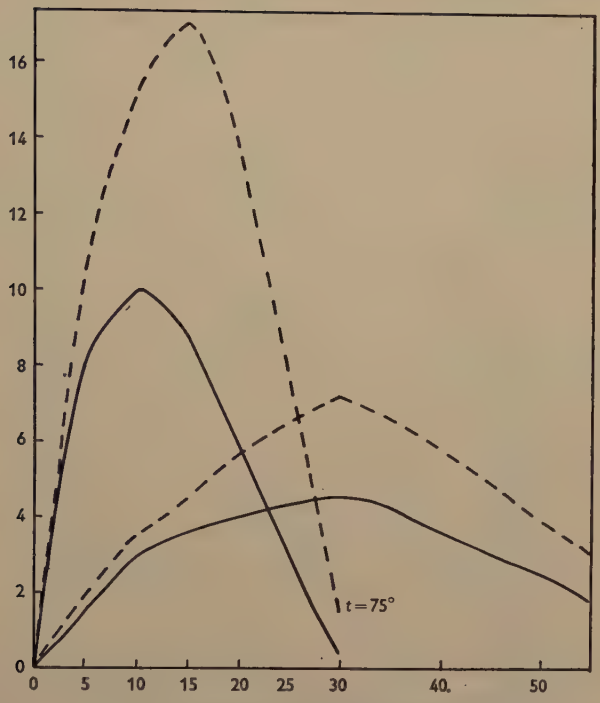


Fig. 3. Äquivalente Endiol/100 Mol. Xylose.
Äquivalente Endiol aus D-Xylose beob. ————,
Äquivalente Endiol aus D-Xylose korr. — — — —.

Isolierung eines p-Nitranilids nach Erwärmen der Xylose auf 92°: 10 g D-Xylose +7 g Bleiacetat wurden in 150 ml Wasser auf 92° erwärmt. Nach Zusatz von 4,5 g NaOH in 50 ml Wasser wurde die Mischung weitere 4 Minuten auf 92° gehalten. Nach Neutralisation wurde abgekühlt und der entstandene Niederschlag mit Wasser, Aceton und Äther gewaschen. Ausbeute 6 g an hellbraunem Bleisalz. Dasselbe wurde, in Aceton aufgeschlämmt, mit 1 ml konz. Schwefelsäure zerlegt. Nach Abfiltrieren des Bleisulfates entstand in der eingedampften Lösung ein öliges Produkt, von wenig Krystallen durchsetzt. Der ölige Anteil wurde mit wenig Wasser aufgenommen, 1/3 davon mit *p*-Nitranilin und Eisessig versetzt. Die über Nacht ausgefallenen Krystalle wurden mit Aceton und Äther gewaschen und erwiesen sich nach Aussehen und Schpt. (230°) als *p*-Nitranilid des Reduktons I.

Die Bildung des Reduktons I aus Xylose ist offenbar stark von der Temperatur und Zeit der Erhitzung in der alkalischen Lösung abhängig. Nach unseren chromatographischen Ergebnissen bildet sich beim Erhitzen auf Temperaturen von 75° und niedriger (3–10 Minuten) Redukton I nur spurenweise; dagegen bilden sich *andere Reduktone* mit vermutlich höherer C-Atomzahl. Xylose liefert nach Erwärmen unter 75° einen Haupt-Flecken Rf 0,36.

Alkalische Behandlung der Maltose

Bei der TR-Titration entspricht 1 Mol Maltose 2 Äquivalenten Endiol[1]. Wie früher bereits betont wurde, ist dieses Ergebnis auffallend, da man nicht annehmen kann,

[1] EULER und H. HASSELQUIST, Dieses Arkiv *1*, nr 40; 1949. — Reduktone, Stuttgart 1950, S. 14.

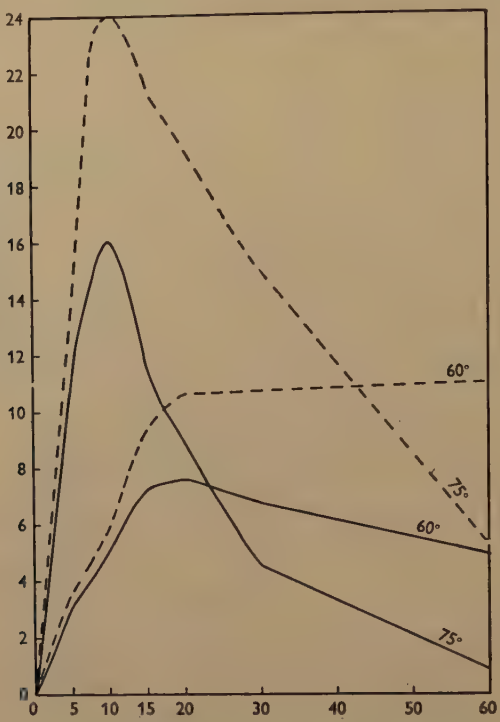


Fig. 4. Äquivalente Endiol/50 Mol Maltose.

Äquivalente Endiol aus Maltose beob. ———,
Äquivalente Endiol aus Maltose korr. — — — —.

dass bei der angewandten Alkali-Konzentration eine Hydrolyse der Maltose eintritt. Wir haben deswegen die reine (nicht-oxydative) Alkaliwirkung mit 0,3 *n*. NaOH untersucht (Fig. 4).

Man sieht, dass bei 10 Minuten mit Maltose ein noch höheres Maximum der Endiol-Bildung erreicht wird als mit Xylose. Es ist möglich, dass diese höhere Reaktionsfähigkeit der Maltose und Xylose im Vergleich zu Glucose mit der Bildung von 2 Endiolgruppen per Mol Zucker zusammenhängt.

Nach der alkalischen Behandlung der Maltose haben wir wiederholt, aber ohne Erfolg, in der Reaktionslösung ein Redukton mit 9 C-Atomen als Bleisalz zu isolieren versucht.[1]

Nach rein alkalischer (nicht oxydativer) Behandlung der Maltose bei 75° (0,3 *n*. NaOH 3 Min.) finden wir bei den papierchromatographischen Versuchen (TR-Entwickler) nur Spuren von Redukton I, dagegen einen Flecken Rf = 0,32, der auf ein höheres Redukton hindeutet.

Erwärmt man die Maltose-Lösung wie auch die Lösungen von Xylose, Lactose und Glucosamin länger als 10 Minuten (also über das Maximum in den Fig. 3–5

[1] Siehe hierzu auch Angaben von F. WEYGAND über das Vorkommen von Triose-Redukton und von anderen Endiolen bei der alkalischen Behandlung von verschiedenen Zuckerarten, 90°, 3 Minuten. (Dieses Arkiv *3*, nr 2; 1950.)

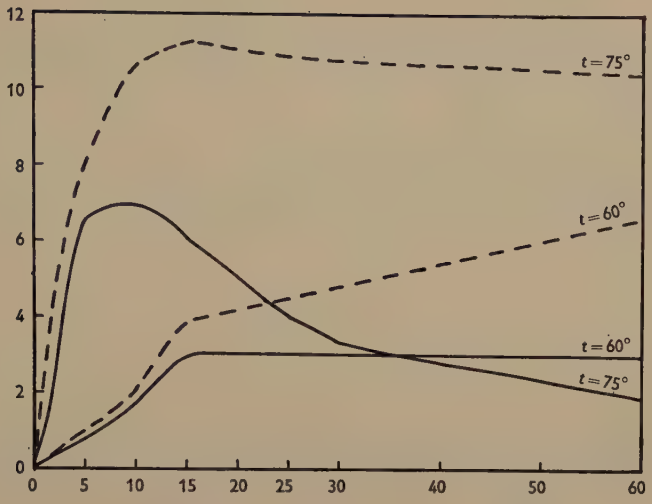


Fig. 5. Äquivalente Endiol/100 Mol D-Glucosamin.

Äquivalente Endiol aus D-Glucosamin beob. ————,
Äquivalente Endiol aus D-Glucosamin korr. — — — —.

hinaus), so nimmt die Stärke der Flecken ab, bis sie nach Erhitzung bis 30 Minuten ganz verschwinden.

Über die Identität der hier erwähnten, durch die Flecken 0,36 und 0,32 nachgewiesenen Reduktone mit den früher beobachteten Reduktonen II und III können wir erst später Mitteilung machen.

Alkalische Behandlung des Glucosamins

Der Umstand, dass in früheren Arbeiten aus Glucosamin kein Redukton erhalten wurde, beruht auf der oben angegebenen Tatsache, dass die Endiol-Bildung qualitativ und quantitativ in recht hohem Grad von der Temperatur, Dauer und NaOH-Konzentration der alkalischen Behandlung abhängig ist. Die in Fig. 5 dargestellte Versuchsreihe zeigt eine Endiol-Bildung an, welche nicht geringer ist, als die mit D-Glucose erhaltene.

Bei der Papierchromatographie (Entwickler TR) wurde mit der 10 Minuten erhitzten Lösung der Flecken Rf = 0,46 erhalten. Im Gegensatz zum Verhalten der Xylose, Maltose und Lactose konnte nach der Alkali-Behandlung des Glucosamins auch bei Temperaturen von 80–85° kein dem Redukton I entsprechender Flecken hervorgerufen werden.

Alkalische Behandlung der Lactose

Da auch Lactose, wie Maltose, eine freie Aldehydgruppe enthält, so konnte vermutet werden, dass auch Lactose bei der TR-Titration 2 Endiole auf 1 Mol Zucker liefern würde. Die diesbezüglichen Ergebnisse besitzen nicht den gleichen Grad der

Sicherheit wie die mit den anderen Zuckern erhaltenen, da der Farbenumschlag bei der Titration unscharf ist. Jedenfalls kann festgestellt werden, dass unter analogen Versuchsbedingungen wie die für D-Glucose und Maltose erwähnten, mehr als 1 Endiol per Mol Lactose auftritt.

Die Ergebnisse dieser Versuche und die daraus zu ziehenden Schlusse werden demnächst an anderer Stelle zusammengefasst werden.

Stockholm, Universität, Institut für organ.-chemische Forschung.

Tryckt den 28 mars 1958

Uppsala 1958. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 12 nr 31

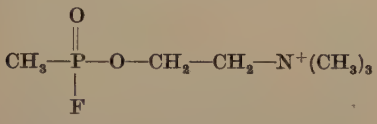
Communicated 22 January 1958 by Karl Myrbäck and Arne Fredga

Organophosphorylcholines and cholinesterases

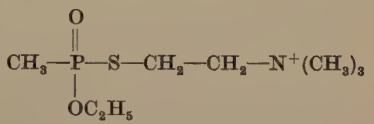
By L.-E. Tammelin

With 7 figures in the text

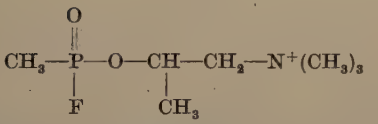
Two types of organophosphorus choline esters have been described in earlier papers (1, 2). They were prepared in order to obtain organophosphorus compounds analogous to acetylcholine.



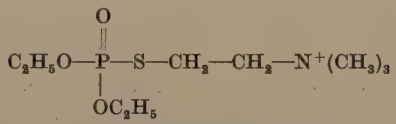
Methyl-fluoro-phosphorylcholine
(MFPCh)



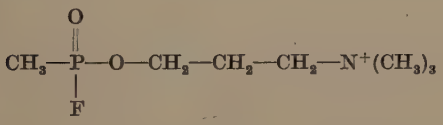
Methyl-ethoxy-phosphorylthiocholine
(MEPtCh)



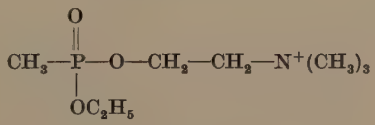
Methyl-fluoro-phosphoryl-β-methylcholine
(MFPβCh)



Diethoxy-phosphorylthiocholine
(DEPtCh)

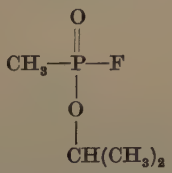


Methyl-fluoro-phosphorylhomocholine
(MFPhCh)



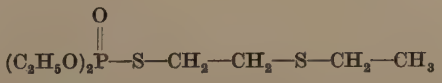
Methyl-ethoxy-phosphorylcholine
(MEPCh)

The compounds are related not only to acetylcholine but also to organophosphorus cholinesterase inhibitors such as



methyl-*iso*-propoxy-phosphoryl fluoride, Sarin[3]

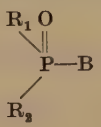
and



iso-Systox[4]

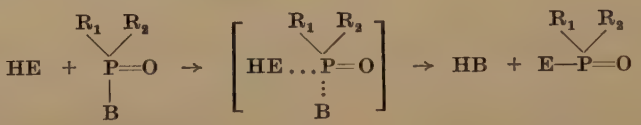
The pharmacodynamic effects of the methyl-fluoro-phosphorylcholines have been referred to inhibition of cholinesterases and direct action on acetylcholine receptors (5, 6). It is very reasonable to assume that the organophosphorus thiocholine esters have a similar action. Strong cholinesterase inhibiting effect has been demonstrated for methyl-fluoro-phosphorylcholines (1) and the thiocholine esters (2) but not in the case of organophosphorylcholines such as MEPCh which are weak inhibitors (2).

G. Schrader (7) has given the following general formula of phosphorylating cholinesterase inhibitors



where B was called acyl.

B is a basic residue such as fluorine, *p*-nitrophenoxy, cyanide or disubstituted phosphoryloxy group. R_1 and R_2 can be alkyl, alkoxy or amido groups. A generally accepted theory by Aldridge (8) concerning the irreversible mechanism of cholinesterase inhibition by organophosphorus compounds is shown in the following formula where HE means cholinesterase.



Aldridge was also able to demonstrate that cholinesterase is diethoxyphosphorylated by both tetraethylpyrophosphate (TEPP) and diethyl-p-nitrophenylphosphate (E 600).

The following is an attempt to demonstrate if and how the compounds listed in the beginning follow the reaction scheme given above. It is also an attempt to give a further description of the inhibitory properties of the presented compounds, of which I_{50} values already have been determined (1, 2), by means of determinations of reaction velocities of the inhibitors with the enzyme. Owing to the special structures of the inhibitors it also seemed reasonable that indirect information about the cholinesterase surface would be obtained.

Experimental

Preparation of methyl-fluoro-phosphorylhomocholine iodide

The synthesis was made according to the methods given in an earlier paper (1). M.p. 82°C Found: C 25.6; H 5.5; I 39.1 Calc. for $C_7H_{18}FINO_2P$ (M = 325.1): C 25.9; H 5.7; I 39.1.

Paper chromatography

The solutions investigated were: 1 % solutions of MEPtCh and DEPtCh in 0.1 M aqueous sodium hydroxide had been stored at 25°C for three hours. 1 % aqueous solutions of the same compounds. It can be calculated from the hydrolysis experiments described below that almost complete hydrolysis occurs in the sodium hydroxide.

Table 1. A compilation of the results obtained by paper chromatography of organophosphorylthiocholines and their alkaline hydrolysates.

Compound	R_F	DPA	NEM	AMo	Compound found
		Developers			
DEPtCh	0.56	+	+	–	DEPtCh
DEPtCh hydrolysate	0.46	+	+	–	thiocholine
	0.41	–	–	+	DEP—OH
	0.22	+	+	–	oxidized thiocholine
MEPtCh	0.47	+	(+)	–	MEPtCh
MEPtCh hydrolysate	0.44	+	+	–	thiocholine
	0.49	–	–	+	MEP—OH
	0.24	+	+	–	oxidized thiocholine

Solvent: *n*-buthanol 8, ethanol 2, acetic acid 2 and water 3 parts by volume. *Reagents:* Dipicrylamine (9) (DPA), (—N—); N-ethyl-maleinimide (10) (NEM), (SH, S—S) and ammonium molybdate + hydrogensulfide (11) (AMo), $\left(\begin{array}{c} O \\ \| \\ -P- \\ | \end{array}\right)$. *Paper:* Whatman 1. Height 35 cm. *Procedure:* Spots of about 10 μl of the solutions (three spots of each) were put on the paper. Descending technique was used at 25°C for five hours. The developers were used as in the references. For results see Table 1.

Apparatus

The electrometric method described by Tammelin (12) was used for the I_{50} determinations in the earlier papers (1, 2). Most of the experiments in this paper have been done with a similar technique. However in determinations of the high reaction velocities between enzyme and methylfluorophosphorylcholines an automatic recording titrator (13) was found to be more suitable. The experiments were done at pH = 7.5 and 25°C. A few experiments were done to demonstrate the usefulness of this set up, and the solutions used, for cholinesterase activity determinations, the results of which are shown in Fig. 1. Curves obtained are in agreement with what is found by other methods. Values are corrected for spontaneous hydrolysis. The same apparatus was also used in the determinations of rate constants of spontaneous hydrolytic cleavage of MEPtCh and DEPtCh and corresponding choline esters. The technique in this last case closely adhered to that used by Larsson (13) in his investigations of Sarin.

I_{50} determinations

The electrometric method (12) was used for the determination of I_{50} and the procedure was the same as in earlier papers (1, 2). (See also Fig. 7.) Source of AChE was human erythrocytes and of BuChE human plasma.

One way to make I_{50} values comparable is to standardize all conditions during the determinations including the incubation time. Another way is to determine the incubation time after

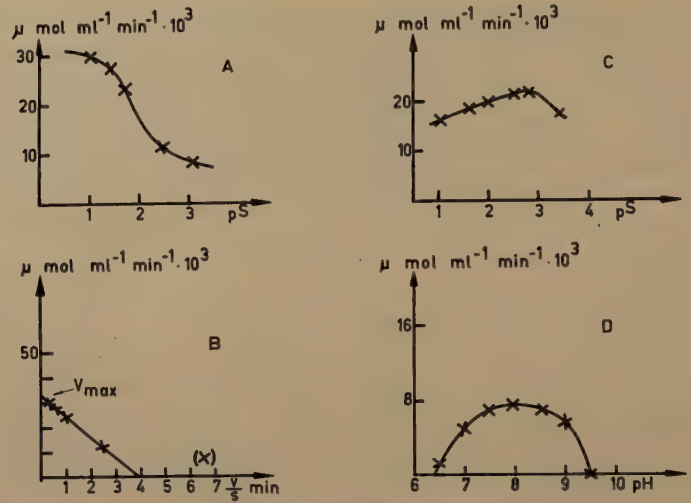


Fig. 1. Studies of the automatic titrator (13) with 0.1 M KCl as solvent and ACh as substrate A and C are activity/pS curves for BuChE and AChE. B shows that A on the whole has the expected form found for BuChE. D shows how activity depends on pH for BuChE.

which no measureable change in enzymic activity is found at inhibitor concentrations close to I_{50} and then work with individual incubation times for the inhibitors. These incubation times shall for every inhibitor closely resemble infinite incubation. The first way is theoretically most attractive if I_{50} values in that way could be considered as kinetic data. However in case of the methyl-fluoro-phosphorylcholines it was hardly possible to perform measurements before the reaction had terminated (Fig. 5). This is due to side reactions such as spontaneous hydrolysis and probably nonspecific reactions with the proteins present and also to the impossibility of making reproducible dilutions with concentrations of the inhibitor lower than about 10^{-11}—M in which case great errors are involved. It was thus decided to use the second alternative. In case of the organophosphorylthiocholines, hydrolysis is slow but after two hours incubation time the enzymic activity was found to be constant.

Reaction velocities of MEPtCh and DEPtCh with cholinesterases

Enzyme solutions: 13.3 mg of plasma fractions IV-6-3. (A. B. Kabi, Stockholm) in 100 ml Michel buffer solution as modified by Tammelin (12). A suspension of 330 mg of freeze-dried electric organ from electric ray (Torpedo oscellata) was homogenized and diluted to 100 ml in Michel buffer solution (12). After centrifugation the supernatant was used. IV-6-3 contains the nonspecific or butyrylcholine esterase (14) (BuChE) and the electric organ the specific or acetylcholine esterase (14) (AChE).

Inhibitor solutions: The following concentrations of iodides of the inhibitors were prepared as aqueous solutions.

Enzyme	DEPtCh molarity	MEPtCh molarity
AChE	10^{-6}	$10^{-7.5}$
BuChE	10^{-7}	10^{-6}

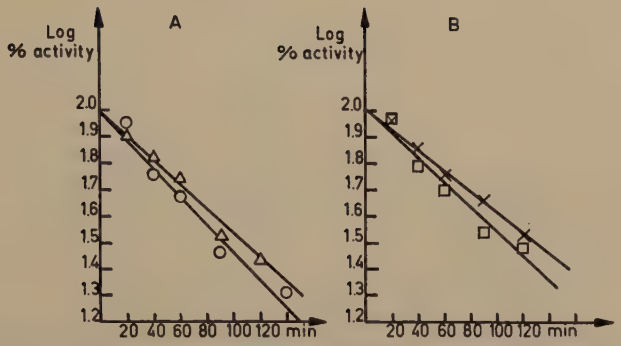


Fig. 2. A shows the reaction velocity of MEPtCh (○) and DEPtCh (△) with AChE. B shows the reaction velocity of MEPtCh (×) and DEPtCh (□) with BuChE. Activity measured by electrometric method (12) with ACh as substrate.

Substrate solution: 0.110 g ACh iodide in 5 ml H_2O.

Reaction mixture: 3 ml of enzyme solution, 2.4 ml of H_2O and 0.6 ml of inhibitor solution. Final pH is 8.

Procedure: A series of reaction mixtures in one of which the inhibitor solution was substituted by water was incubated at 25°C. After various lengths of time 0.6 ml of the substrate was added and the enzymic activities determined electrometrically (12). Activities are expressed as percent of uninhibited enzyme. For results see Fig. 2 and Table 2. Straight lines are obtained if log (percent activity) is plotted against time. Half life and k_2 are calculated as in the experiments with methyl-fluoro-phosphorylcholines.

Reaction velocities of MFPβCh and MFPhCh with cholinesterases

Tests with a similar technique as above showed that concentrations of MFPβCh and MFPhCh which cause complete inhibition of the cholinesterases result in reaction velocities which are

Table 2. Reactivities of organophosphorylcholines and related compounds expressed as second order velocity constants for reaction with hydroxyl ions and cholinesterases. pI_{50} values are given for comparison.

	Alkaline hydrolysis: k_2 1 mol^{-1} sec^{-1} 25°C	Phosphorylation of cholinesterase: k_2 1 mol^{-1} sec^{-1} 25°C		pI_{50}	
		AChE electric ray	BuChE IV-6-3	AChE human erythrocytes	BuChE human plasma
MFPCh	935[13]	—	—	10.0±0.2[1]	8.4±0.2[1]
MFPβCh	381[13]	$(6\pm1)\cdot10^{3}$ *	$(6\pm1)\cdot10^{4}$ **	8.4±0.2[1]	8.4±0.2[1]
MFPhCh	305[13]	$(3\pm1)\cdot10^{4}$ *	$(1.9\pm0.2)\cdot10^{5}$ **	11±0.5	8.4±0.2
Sarin	26[13]	$(6\pm1)\cdot10^{3}$ *	$(7\pm1)\cdot10^{3}$ **	8.8±0.2	8.4±0.2
MEPtCh	0.20	$(7\pm1)\cdot10^{4}$ ***	$(2\pm0.5)\cdot10^{3}$ ***	9.1±0.2[2]	7.9±0.2[2]
DEPtCh	0.70	$(2\pm0.5)\cdot10^{3}$ ***	$(2+0.5)\cdot10^{4}$ ***	8.4±0.2[2]	8.9±0.2[2]
MEPCh	0.040	—	—	<4	<4
DEPCh	0.043	—	—	<4	4.5

* Inhibition process at a substrate concentration of $3.12\cdot10^{-3}$.
** Inhibition process at a substrate concentration of $1.87\cdot10^{-2}$.
*** Inhibition process without substrate.

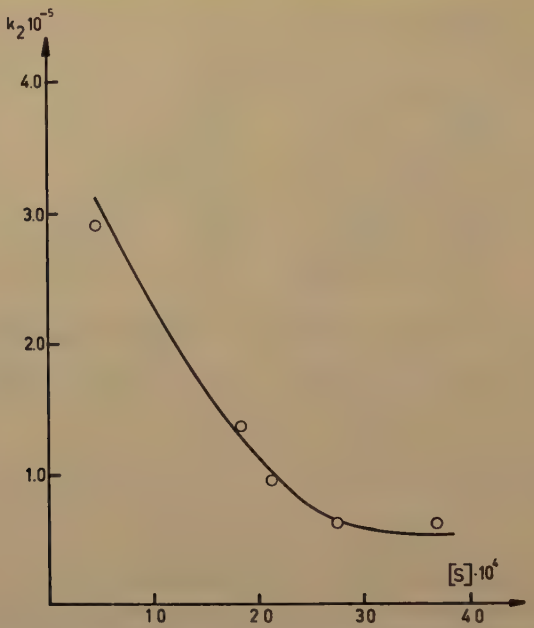


Fig. 3. Reaction velocity of MFPβCh with AChE expressed as second order velocity constant is shown as function of the substrate concentration. Inhibitor is added after the substrate and reaction velocity determined as shown in Figs. 4 and 5.

too fast to be followed in that way. It was thus decided to protect the enzymes with the substrate before the addition of inhibitor and to use the automatic titrator (13).

Enzyme solutions: 25mg of IV-6-3 (BuChE) in 100 ml 0.1 M aqueous potassium chloride. A suspension of 500 mg of homogenized freeze-dried electric organ (AChE) from electric ray in 100 ml 0.1 M aqueous potassium chloride. The solution was filtered through Munktell No 3 filter paper before use.

A. *Reaction velocity and inhibitor concentration:*

Enzymes: AChE, BuChE.

Inhibitor solutions: The following molarities in 0.1 M potassium chloride were prepared:

BuChE: MFPβCh: $1.6 \cdot 10^{-5}$; $4 \cdot 10^{-6}$; $4 \cdot 10^{-7}$ M.
MFPhCh: $4 \cdot 10^{-6}$; $2 \cdot 10^{-6}$; $4 \cdot 10^{-7}$ M.
Sarin: $5 \cdot 10^{-5}$

AChE: MFPβCh: $2 \cdot 10^{-5}$; $1.2 \cdot 10^{-5}$ M.
MFPhCh: $4 \cdot 10^{-6}$ M.
Sarin: $5 \cdot 10^{-5}$ M.

Because of the fast spontaneous hydrolysis the solutions must be freshly prepared. MFPCh hydrolysis is too fast to allow preparation of solutions reproducible enough for these experiments. (For example of results see Fig. 5.)

Substrate solutions

BuChE: 0.683 g ACh iodide in 100 ml 0.1 M potassium chloride.

AChE: 0.114 g ACh iodide in 100 ml 0.1 M potassium chloride.

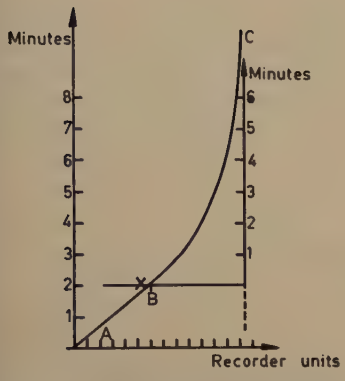


Fig. 4.

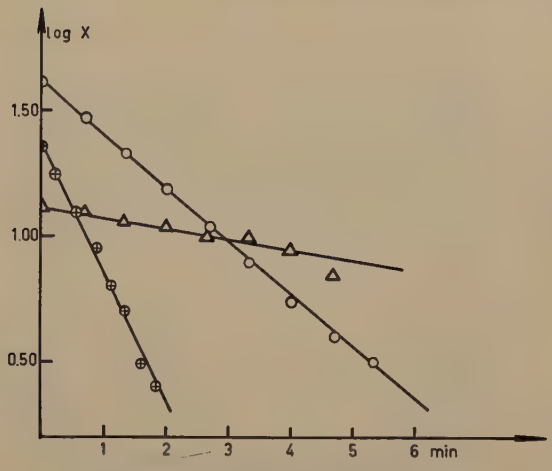


Fig. 5.

Fig. 4. Determination of reaction velocity between methylfluorophosphorylcholines and substrate (ACh) protected cholinesterase. Automatic titrator recorder paper moves along time axis. Recorder units are proportional to sodium hydroxide consumption and to amount of acid formed. A–B shows the uninhibited process. Inhibitor is added at B. B–C shows how activity decreases after the addition.

Fig. 5. Velocity of reaction between MFPhCh and BuChE protected by $1.9 \cdot 10^{-2}$ M ACh. Half life of the enzyme is found as half life of x. x as function of time is found in the x/time coordinate system in recordings such as illustrated in Fig. 4. Different inhibitor concentrations were used (△) 10^{-8}M, (○) $0.5 \cdot 10^{-7}$M and (⊕) 10^{-7}M.

B. *Reaction velocity and substrate concentration:*

Enzyme: AChE.

Inhibitor solution: MFPβCh $1.25 \cdot 10^{-6}$ M in 0.1 M potassium chloride.

Substrate solutions: 0.114 g ACh iodide in 100 ml 0.1 M KCl solution. For final concentrations and result see Fig. 3.

Experiments with automatic titrator:

		BuChE	AChE
Reaction mixture:	Substrate	30 ml	30 ml
	0.1 M KCl	6 „	8 „
	Enzyme	3 „	1 „
	Inhibitor	1 „	1 „

pH was 7.5 and temperature 25°C.

Procedure: The reaction mixture without inhibitor was titrated in the automatic titrator for some minutes at pH = 7.5 during which a linear relation between sodium hydroxide (0.025 M) consumption and time was recorded (A–B in Fig. 4). The inhibitor solution was added when this line was established and the following decreasing activity (B–C in Fig. 4) is recorded continuously.

Calculations: The slope of A–B in Fig. 4 gives uninhibited enzymic activity. Such lines were used for calculation of results in Fig. 1. Inhibitor is added at B. The slope of the tangent

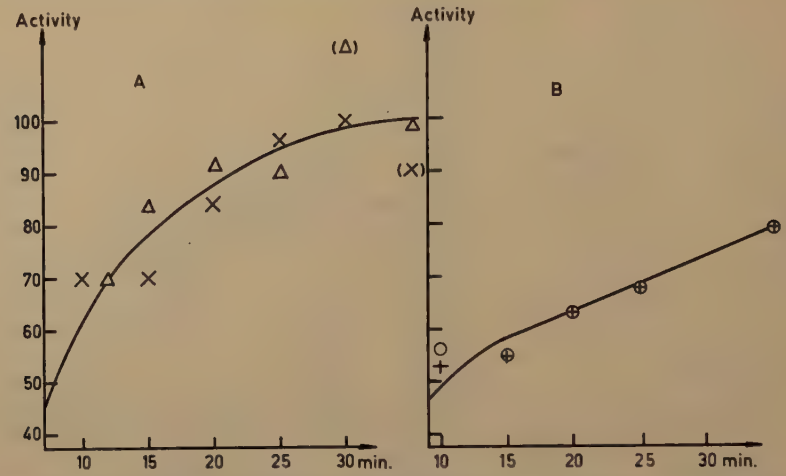


Fig. 6. Reactivation with PAM of inhibited AChE. Both diagrams show enzymic activity (12) as function of reactivation time. In A activity was inhibited to zero by TEPP (×) and DEPtCh (△). In B activity was inhibited to zero by Sarin (+) and MiPPtCh (○). Identity of activity/time curves, two by two, supports the phosphorylation theory (8).

to B–C in the x/time coordinate system shows the activity during the inhibition process. As time axis the asymptote of B–C was chosen. If log x is plotted against time a straight line is obtained (Fig. 5) which shows that B–C is an exponential function. Due to the exponential form the half life of enzymic activity, which is expressed by the tangent to B–C, equals the time for fifty percent reduction of x.

If log x is plotted against time as in Fig. 5 the half life of the enzyme $t_{1/2}$ is found as the time interval corresponding to a change of 0.3 in log x. The second order velocity constant k_2 in $\frac{-d\,(\text{activity})}{dt} = k_2$ [activity] [I], where [I] is inhibitor concentration, is calculated from

$$k_2 = \frac{0.693}{t_{1/2} \cdot [\text{I}]} \quad 1 \text{ mol}^{-1} \text{ sec}^{-1}.$$

Organophosphorylthiocholines and phosphorylphosphatases

The determinations were made by Augustinsson (private communication) according to methods given in his papers (15, 16).

Reactivation experiments

The method of Davies and Green (17) and the electrometric method (12) was used.

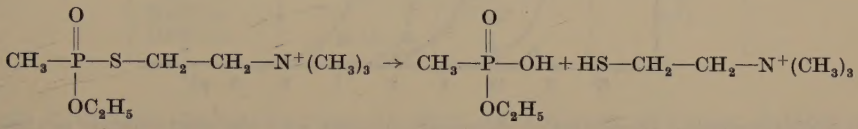
Inhibition process: Washed human erythrocytes diluted to blood volume were inhibited by 10^{-6} M TEPP and 10^{-6} M DEPtCh in separate vessels. The same was done with 10^{-6} M sarin and 10^{-6} M methyl-*iso*-propoxy-phosphorylthiocholine (MiPPtCh). The inhibited erythrocytes were washed three times with saline.

Reactivation process: Erythrocytes diluted to twice the blood volume were treated with $2.5 \cdot 10^{-4}$ M 2-pyridine aldoxime methiodide (PAM). Temperature 25°C.

Activity determination: Samples of the reactivated erythrocytes were taken at time intervals shown in Fig. 6. Enzymic activity was determined as in the I_{50} experiments. Final dilution of erythrocytes was twenty times of blood volume.

Results

The basic residue B in Schraders formula is fluorine in methylfluorophosphorylcholines and thiocholine residue in MEPtCh, DEPtCh and related compounds. In case of the methyl-fluorophosphorylcholines it has been shown by alkaline hydrolysis that fluorine is split off much easier than any other group (13). MEPtCh and DEPtCh are hydrolyzed in alkaline solution. Second order velocity constants are given in Table 2 and the main course of the process has been shown to be according to



which is seen by the paper chromatography experiments (Table 1). Moreover the thiocholine and oxidized thiocholine are recognized by their found R_F values 0.45 and 0.23 which have also been found by Heilbronn[18] in experiments with thiocholine and carboxylic esters of thiocholine.

An automatic titrator (13) has been tested for cholinesterase activity determinations (Fig. 1) and a method for determinations of rapid inhibition reactions (half lifes down to 1 min) has been worked out (Fig. 4 and 5).

The organophosphorylcholines have been shown to be competitive inhibitors. In case of the thiocholine esters the substrate concentration used protected both enzyme preparations completely up to 10^{-5}M solutions of the inhibitors. The methylfluorophosphorylcholines which react extremely fast, especially with AChE, give decreases, although slower, of enzyme activity even in the presence of substrate. The protective effect was utilized to retard the reaction with the enzymes to a measurable velocity. Fig. 3 shows an example of the protective effect of different substrate concentrations.

It has been shown that the organophosphorylcholines of both types react with the enzyme according to pseudo-first order kinetics in the inhibitor concentration range investigated. Second order velocity constants are given in Table 2. Comparisons should only be done between values with the same number of asterisks. The organophosphorylcholines of the type MEPCh and DEPCh are weak inhibitors and give low toxicity (Table 3) but give instantaneous inhibition. The later compounds are probably reversible inhibitors which may follow from the relatively slow hydrolysis of the choline group shown in Table 2. If there is any reaction except ion exchange with the enzyme it is likely to be very slow.

Reaction velocity between AChE or BuChE and MFPCh and MFPhCh is extremely high as judged from the I_{50} values and the kinetic data on MFPhCh compared to methyl-*iso*-propoxyphosphoryl fluoride (Table 2). One consequence of this high reaction velocity is that no difference in pI_{50} could be found if incubation time was varied between 15 and 120 min or if a human serum preparation of the same activity as the purified serum preparation IV-6-3 was used.

Reaction velocity between AChE or BuChE and the thiocholine esters is comparatively slow and of the same order of magnitude as Aldridge (8) found for E 600 (Table 2). I_{50} of the thiocholine esters showed thus dependence of incubation time but was also independent of the purity of the enzyme preparation in the same

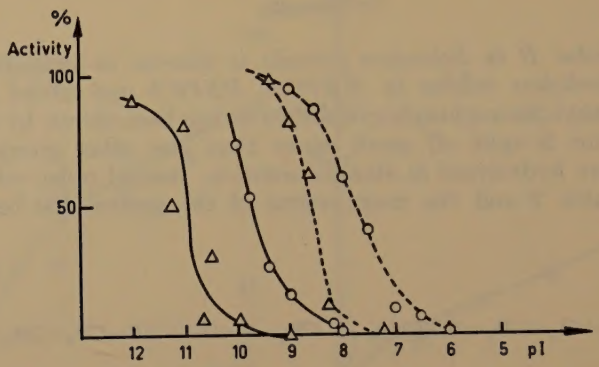


Fig. 7. Activity/inhibitor concentration diagrams obtained with the electrometric method (12) illustrating the possibility of selective inhibition of AChE. (△) show results with MFPhCh and (○) with MEPtCh. —— show results with AChE and – – – with BuChE.

way as the methyl-fluoro-phosphorylcholines. Stability against phosphorylphosphatases found in the experiments according to Augustinsson (15, 16) seems to be the explanation.

The reactivation experiments with PAM (See Fig. 6) indicate that *the enzyme is diethoxyphosphorylated by TEPP and DEPtCh or methyl-iso-propoxyphosphorylated by Sarin and MiPPtCh.* Thus these experiments demonstrate that thiocholine is split off during the inhibition process. These conclusions follow from the identical reactivation velocities found for TEPP and DEPtCh inhibited AChE. Another reactivation velocity, though coinciding for the two, was found for methyl-*iso*-propoxyphosphoryl fluoride and methyl-*iso*-propoxyphosphorylthiocholines.

All data found indicate that the reaction scheme given by Aldridge (8) for organophosphorus inhibitors is followed by the present compounds.

Organophosphorylthiocholines are unaffected by phosphorylphosphatases from human plasma (IV-1 AB Kabi, Stockholm). Methylfluorophosphorylcholines show too fast spontaneous hydrolysis for demonstration of a phosphorylphosphatase effect.

Two of the inhibitors *MFPhCh and MEPtCh have been shown to be rather good as selective inhibitors of AChE* (Fig. 7). Such inhibitors are of interest in e.g. histochemical work particularly since only reversible selective inhibitors of AChE have been described for this purpose (19).

Discussion

The I_{50}-values, for phosphorylating inhibitors, are not simply an expression for their affinity for, or reaction velocity with, the enzyme. It has been pointed out by Jandorf (20) *et al.* that side reactions are the only reasons why organophosphorus inhibitors do not give complete inhibition at all concentrations above the molar concentration of active enzyme groups. MFPhCh has been shown to inhibit human erythrocyte AChE (haemolyzed erythrocytes diluted to 1/30 of blood volume) to fifty percent at a [I] of about 10^{-11}. A molar concentration of about twice as many active groups in such an enzyme preparation is thus a probable maximum value. It might be lower because of a systematical loss of inhibitor

Table 3. Toxicities of organophosphorylcholines determined by intraperitoneal injection in white mice by Fredriksson. Toxicities of methylfluorophosphorylcholines have been published (5, 6), others are unpublished results.

Compound	LD_{50} mg/kg	Compound	LD_{50} mg/kg
MFPCh	0.10	DEPCh	495
MFPβCh	0.07	MiPPtCh	0.12
MFPhCh	0.05	MiPPCh	210
MEPtCh	0.03	Sarin	0.42
MEPCh	375	TEPP	0.74
DEPtCh	0.14		

due to hydrolysis and adsorption at glass walls during dilutions. Inhibitors are used in considerable excess to 10^{-11} M in most I_{50} determinations published (pI_{50} seldom passes 10) and Jandorf (20) has pointed out that hydrolysis of the inhibitor is one side reaction to consider, others are reactions with other groups than the active in the proteins present. Methylfluorophosphorylcholines show considerable spontaneous hydrolysis as seen from the k_2 values in Table 2 which may be one of the main reasons for the excess needed. The thiocholine esters are comparatively stable to spontaneous hydrolysis and unaffected by phosphorylphosphatases, facts which make it more complicated to understand the termination of the enzyme-inhibitor reaction in two hours. Only explanations such as losses due to adsorption at other places than the active centers seem to remain, e.g. at the proteins.

The preceding reasoning together with physiological factors such as distribution in the body, excretion and metabolism makes correlations of I_{50} values to toxicity in organophosphorus compounds rather doubtful. In homologous series, where correlation is expected, or series of closely related compounds such a correlation has been found (21) but in general there seems to be little hope of finding a correlation.

However I_{50} values seem to be a better basis for a rough prediction of toxicity than the reaction velocity with the enzyme. In Table 3 it is shown that high toxicities are found both among the highly reactive methylfluorophosphorylcholines and among the slowly reacting thiocholine esters. The first group of compounds are toxic due to their reactivity and the second due to stability against side reactions. In both types of compounds affinity for ACh chemoreceptors are likely to contribute to the intoxication picture.

It seems very reasonable that the potent inhibitors among the organophosphorylcholines react with the enzyme quite in the same way as substrates; however deacylation of the enzyme is impossible in case of the organophosphorus compounds. The extraordinarily strong inhibiting properties seem also to indicate that an anionic site of the enzyme is involved in formation of the intermediate step which might be a transition state. The high potency of these inhibitors seems thus to support the theories about the cholinesterase surface put forward by Wilson (22).

The results in this paper confirm the predictions made in the two earlier papers (1, 2) that organophosphorylcholines due to their structural similarity with ACh show strong cholinesterase inhibiting and toxic properties.

SUMMARY

The kinetics of the inhibition of cholinesterases with methyl-fluoro-phosphorylcholines and organophosphorylthiocholines has been studied. Reactivation of cholinesterase inhibited by organophosphorylthiocholines by PAM was used in order to demonstrate the phosphorylation of the enzyme. Thiocholine was found to be split off during the spontaneous alkaline hydrolysis of organophosphorylthiocholines and determination of the second order velocity constant showed that hydrolysis is relatively slow. However, it seems thus likely that the thiocholine residue can be considered as the reactive group. Methyl-fluoro-phosphorylcholines showed extremely fast reactions with cholinesterases whereas methyl-ethoxy-phosphorylthiocholine and related compounds showed slow reactions. Methyl-*iso*-propoxyphosphorylthiocholine and methyl-*iso*-propoxyphosphoryl fluoride inhibited cholinesterase gave identical reactivation velocities and the same was found if tetraethylpyrophosphate and diethoxyphosphorylthiocholine were compared. These results show that the inhibitors inhibit through phosphorylation. I_{50} values of phosphorylating inhibitors are discussed, also in relation to toxicities which are given for some organophosphorylcholines.

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